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Consequences of fetal exposure to analgesics for germ cells

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Declaration

The experimental work in this thesis is the sole work of the author except where acknowledgment has been made. These studies have not been submitted in support of another degree or qualification at the University of Edinburgh, or any other institute

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Abstract

Despite the general advice of avoiding medication during pregnancy, the majority of pregnant woman use one or more 'over the counter' analgesics. During the last few years there has been growing evidence that analgesic exposure, such as paracetamol, ibuprofen or indomethacin, during pregnancy can have detrimental effects on rodent and human fetal gonads. The majority of previous studies have focused in alterations in testosterone production and male reproductive disorders. However, few studies have analysed the effect of these analgesics on fetal germ cells and possible consequences on fertility.

During my thesis, I first focused on the effect of paracetamol and indomethacin exposure during pregnancy on rat fetal gonads. These showed that both paracetamol and indomethacin are able to alter the expression of genes important for fetal gonad and germ cell development.

Previous studies on germ cells and analgesics have focused on rat models, but there is a lack of similar studies performed in human models. Therefore, I investigated the consequences of exposure of therapeutically relevant doses of paracetamol and ibuprofen on human gonads, with a special attention to the germ cells. Fetal gonads from the 1st and 2nd trimester were used in two different models: hanging drop cultures for 1st trimester testes and ovaries and a xenograft system for 2nd trimester fetal testes. Fetal gonad culture in the presence of paracetamol or ibuprofen reduced AP2γ^{+} (gonocyte) GC number in both 1st trimester fetal testes (22-28% reduction) and ovaries (43-49% reduction). 2nd trimester fetal testes were exposed to three different regimes, 1 or 7 days paracetamol and 7 days ibuprofen, which led to reductions of 17% and 30%, respectively in AP2γ^{+} GC number for paracetamol and a 53% reduction in total germ cell number for ibuprofen.

The last part of the project was focused on determining the mechanism of action of paracetamol and ibuprofen, with special attention to alterations in the prostaglandin E2 pathway. For these studies, a human GC tumour-derived cell line (NT2) that exhibits fetal GC characteristics was used. Exposure to analgesics or prostaglandin E_2 (PGE_2) antagonists reduced NT2 cell number. Furthermore, exposure to PGE_2 agonists prevented paracetamol-induced NT2 cell loss. Following roles of prostaglandins described in the literature, I focused on the study of germ cell pluripotency and effects on epigenetic regulatory genes. Both analgesics and PGE_2 antagonists reduced expression of GC pluripotency genes (*AP2γ*, *OCT4*, *NANOG*) in NT2 cells and altered expression of *DNMT3a* and *3b*, *TET1* and *EZH2*, key genes that regulate DNA and histone methylation. To demonstrate the in vivo translatability of these gene expression effects, I investigated the effects of paracetamol exposure on their expression in vivo and in vitro in rat fetal ovaries and testes and showed changes comparable to those induced in NT2 cells.

The studies presented in this thesis demonstrate that common analgesics can alter fetal germ cell development. The findings show similarities among the different models and translatability of in vitro findings to in vivo and across species. My results also demonstrate that the effect of analgesics are a consequence of alterations in PGE_2 action, and raise concerns that similar effects might occur following analgesic use in human pregnancy, including potential next generation effects.

Lay summary

Pregnant women are generally advised to avoid any kind of medication during pregnancy. However, the majority of them use one or more 'over the counter' analgesics, which are also sometimes prescribed by doctors. These analgesics, including paracetamol, ibuprofen or aspirin, have been shown to be associated with increased risk of autism and allergies and other consequences. Moreover, analgesic exposure of fetal testes has been shown to reduce production of the male sex hormone (testosterone), which can result in male reproductive disorders such as undescended testes or penis developmental abnormalities. Recent experimental studies using laboratory animals have also revealed that analgesics can reduce the number of fetal germ cells, which are the cells that will become oocytes (eggs) and sperm. Furthermore, this had detrimental effects on fertility when females exposed to analgesics while fetuses grew up to become adults.

There is no ethically acceptable way of directly investigating if similar germ cell effects occur in the human as a consequence of fetal exposure to analgesics. Therefore, this thesis focused on analgesic effects on germ cells and the possible mechanisms involved using a variety of model systems using human and rat fetal gonad cultures or xenografts, and one in vivo rat model (pregnancy exposure to analgesics). This was complemented by a cellular model (NT2 cells) derived from a germ cell tumour, which shows characteristics of fetal germ cells.

The rat models showed that paracetamol and ibuprofen have some effects on germ cell development and alter activity of genes important for germ cell development. In order to establish human relevance of the rat findings, 1st and 2nd trimester fetal human gonads were analysed. 1st trimester ovaries and testes were exposed to amounts of paracetamol and ibuprofen that result from its normal use in humans. 2nd trimester gonads were exposed to the same regimes, plus an extra experiment with only 1 day paracetamol exposure. All treatments caused a reduction in germ cell number as had been found earlier in rats. The NT2 cell model was then used to study

how analgesics cause their effects on germ cells, and this revealed that the effects resulted from alterations in the activity of prostaglandin E2.

As a result of these studies, concerns are raised that use of analgesics by pregnant women may cause changes to germ cells in the fetus (whether male or female) which might reduce fertility or reproductive lifespan in adulthood in exposed (female) fetuses, or cause alterations to the germ cells that might affect the children that they will give rise to

Published articles relating to this thesis

- Hurtado-Gonzalez, P. *et al*. Exposure to acetaminophen and ibuprofen affect fetal germ cell development in both sexes in rodent and human. (Submitted)
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- Dean, A. *et al*. Analgesic exposure in pregnant rats affects fetal germ cell development with inter-generational reproductive consequences. Scientific report (2016).

Presentations relating to this thesis

- Pablo Hurtado-Gonzalez, Rod Mitchell, Richard Anderson and Richard M Sharpe. Exposure to acetaminophen and ibuprofen affect fetal germ cell development in both sexes in rodent and human. Gordon research conference in Germline stem cell biology, Hong Kong, China, June 2017. (poster)
- Pablo Hurtado-Gonzalez, Rod Mitchell, Richard Anderson and Richard M Sharpe. Effect of acetaminophen exposure on germ cells in the human fetal gonad; evidence for epigenetic effects? Gordon research conference in mammalian reproduction, New Hampshire, USA, August 2016. (Poster)
- Pablo Hurtado-Gonzalez, Sander Van der Driesche, Sheila MacPherson, Chris Mckinnell, Richard Anderson and Richard M Sharpe. Effect of analgesic exposure on the epigenetic machinery of germ cells in the fetal rat testis. European testis workshop, Rennes, France, June 2016. (presentation)
- Pablo Hurtado-Gonzalez, Sander Van der Driesche, Sheila MacPherson, Chris Mckinnell, Richard Anderson and Richard M Sharpe. Effect of analgesic exposure during pregnancy on the epigenetic machinery of fetal gem cells in rat. Copenhagen Workshop in endocrine disruptors, Copenhagen, Denmark, April 2015. (Poster)

Abbreviations

5-CaC	5-carboxylcytosine
5-mc	5-methylcytosine
5-hmc	5-hidroximethylcytosine
5-fc	5-formylcytosine
ADHD	Attention deficit/hyperactivity disorder
AMH	Anti-Müllerian hormone
AGD	Anogenital distance
AGD_{AP}	Anogenital distance (Ano-penile)
AGD_{AS}	Anogenital distance (Ano-scrotal)
ALDH1	Aldehyde dehydrogenase 1 family
B2M	Beta-2-microgobulin
BER	Base excision repair
BMP	Cyclic adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
CBX	Chromobox-domain
CGIs	CpG islands
ChIP	Chromatin inmmunoprecipitation
CI	Confidence interval
CO_2	Carbon dioxide
COX	Cyclooxygenase
CYP	Cytochrome P450
DACT	Dishevelled Binding Antagonist of Beta Catenin
DAZL	Deleted in Azoospermia Like
DHT	Dihydrotestosterone
DMRT1	Doublesex and mab-3
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSD	Disorders of sex development
DTT	Dithiothreitol
e	Embryonic day
EGFR	Epidermal growth factor receptor
EP	Prostaglandin receptor
ESC	Embryonic stem cell
EZH2	enhancer of zeste 2
Fgf9	Fibroblast growth factor 9
FOXL2	ForkheadboxL2
FOXO3	Forkhead box O3
GC	Germ cell
GD	Gestational day
GW	Gestational week
H2AK119ub1	Mono-ubiquitination of H2A on lysine 119
H3K27me3	Tri-methylation of the lysine 27 of the histone 3

H3K4me3	Tri-methylation of the lysine 4 of the histone 3
H3K9me2	Di-methylation of the lysine 9 of the histone 3
HCL	Chloride acid
HOX	Homeotic genes
HR	Hazard ratio
ICR	Imprinting control region
INSL3	Insulin-like growth factor 3
JMJD	Jumonji domain-containing protein
Kg	Kilogram
KO	Knock out
LSD1	Lysine-specific histone demethylase 1
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
Mg	Miligramo
NHS	National health service
NSAID	Non-steroidal anti-inflammatory drug
MPW	Masculinization programming window
OCT4	octamer-binding transcription factor 4
OR	Odd ratio
PARP	Poly (ADP-ribose) polymerase
Pax2	Paired box 2
PBS	Phosphate buffered saline
PcG	Polycomb group
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PGCLC	PGC-like cell
PGD	Prostaglandin D
Pgds	Prostaglandin D synthetase
PGE	Prostaglandin E
PGF	Prostaglandin F
PGI	Prostaglandin I
PRC	Polycomb repressive complexes
PREs	Polycomb response elements
PTM	Post translational modifications
qPRC	Quantitative Polymerase chain reaction
RA	Retinoic acid
RFTS	Replication foci targeting sequences
RING	Really interesting new gene
RNA	Ribonucleic acid
R-SPO1	R-spondin 1
Stra8	Stimulated by retinoic acid 8
Sf1	Steroidogenic factor 1
SP	Specificity protein

SRY	Sex determining region of the Y chromosome
SUMO	Small ubiquitin-related modifier
SUZ12	Suppressor of zeste 12
TBP	TATA-box binding protein
TDG	Thymine DNA glycosylase
TDS	Testicular dysgenesis syndrome
TET	Ten-eleven translocase
TGCC	Testicular germ cell cancer
TGF-β	Transforming growth factor-β
tRNA	Transfer RNA
TREs	Trithorax response elementes
TrxG	Trithorax group
UV	Ultraviolet
VGEF	Vascular endothelial growth factor
WNT4	Wingless type MMTV integration site family member 4
WT	Wild-type
WT1	Wilms tumour protein 1
XIST	X-inactive specific transcript
µM	Micro molar

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Chapter 1: Literature review

1.1 Introduction

During the last decades there has been increasing recognition of the importance of fetal development for lifelong good health or disease. Small abnormal modifications in specific pathways can result in a spectrum of conditions. Some of these modifications can be mild and may not be diagnosed or noticed (Bonde et al., 2017). On the other hand, some can cause severe abnormalities, even fetal death. There are several studies which show links between occurrence of these abnormalities and different factors during pregnancy, such as diet, lifestyle, environment, and also drug intake (Gabrielsen & Tanrikut, 2016; Panchenko et al., 2016). Many disorders have been associated with alterations during fetal development and reproductive disorders are examples of this. In males, this is known as Testicular Dysgenesis Syndrome (TDS). Yet little attention has been paid to whether or not the germ cells present in the fetal gonads might also be affected by fetal programming. One important issue, also affecting TDS, is that the actual causes of this fetal abnormal development are essentially unknown.

Women are generally advised to avoid taking any kind of medication during pregnancy. However, the majority of pregnant women take one or more analgesics for pain/fever relief during pregnancy. 65% of pregnant woman take paracetamol (acetaminophen), but also non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen (15% of pregnant women) (Werler et al., 2005). This is due to the fact that they are perceived as being safe and are widely used in the normal population without medical prescription (Werler et al., 2005).

Some recent studies have shown various effects on the fetal gonads as a consequence of analgesic exposure. As an example, a recent study showed how several analgesics can affect hormone production by the human fetal testis in vitro (Mazaud-Guittot et al., 2013). One of those is paracetamol, which is able to cross the

placenta into the fetus (Weigand et al., 1984; Ma et al., 1989). On the other hand, different studies in mice suggest that NSAIDs can interfere with fetal masculinization (Gupta, 1989, Gupta and Goldman, 1986) or inhibit steroidogenesis and/or insulin-like factor 3 production (Chalmey et al., 2013; Kristensen et al., 2011; Kristensen et al., 2012), studied also in the adult human male (Albert et al., 2013).

Recent studies in the rat showed that exposure of pregnant rats to indomethacin (an NSAID), or paracetamol at human-relevant doses, alters fetal germ cell (GC) development (Dean et al., 2016). The same study showed that mating of analgesic-exposed (F1) males or females with unexposed controls produced an F2 generation of females with reduced ovarian size and altered follicle development. This surprising data support the idea of epigenetic modifications produced by both analgesics and raise the possibility that other biological effects could have occurred, as only the gonads have so far been studied.

1.2 Analgesic use in pregnancy and male reproductive development

This section is adapted from ¨Hurtado-Gonzalez and Mitchell. Analgesic use in pregnancy and male reproductive development. Curr Opin Endocrinol Diabetes. 24(3):225-232 doi: 10.1097/MED.0000000000000338 (2017)

(Thesis pages 2 to 18 and Appendix 1)

Due to the lack of studies, pregnant women are advised to avoid using medications. NHS recommends to speak with a doctor before any use of medications (http://www.nhs.uk/Conditions/pregnancy-and-baby/Pages/medicines-in-pregnancy.aspx). Nowadays drug intake during pregnancy is highly reduced, although there are important exceptions. In the case of prescribed drugs, their use depends on the specific needs of the patient. Some drugs, particularly for chronic conditions such as psychiatric diseases, opioids for chronic pain or antidepressants are sometimes advised (Mitchell et al., 2011; Källén & Reis, 2016). In contrast, the intake of ‘over the counter’ drugs depends more on the mother and it can be based

on a lack of knowledge. Deficiency in the information given to prospective parents can create a low awareness about the intake of these drugs. However, some studies focused on call centres showed that many future parents are concerned about the intake of different chemicals during pregnancy, especially paracetamol and ibuprofen (Campbell et al., 2016); (Lau et al., 2016).

1.2.1 Analgesic use during pregnancy

As outlined earlier, the majority of women take one or more analgesics such as paracetamol (acetaminophen) or NSAIDS, at some point during their pregnancy (Werler et al., 2005). A Large Danish study (n=46500) reported analgesic use in 55% of pregnant women (Jensen et al., 2010), whilst a US study (n=10533) reported 65% of pregnant women used paracetamol (15% in combination with ibuprofen) (Werler et al., 2005). A smaller (n=895) French study reported an even higher frequency of analgesic use (81%) in pregnant women (Philippat et al., 2011). Furthermore, the overall consumption of analgesics has increased significantly in the majority of European countries during the past 20 years (Kristensen et al., 2016).

Drugs have been traditionally considered as highly permeable to the placenta. This organ has different mechanisms to transfer a diverse variety of molecular structures from the maternal to foetus blood. These mechanisms include different plasma membrane carriers (Vähäkangas and Myllynen., 2009), export pumps (Stulc J., 1997), as well as biotransforming enzymes within others. This characteristic of permeability has been the base of the general recommendation of limiting the drug use during pregnancy (Marin et al., 2004). The analgesics studied in these thesis are able to cross the placenta and hence have the potential to cause direct effects on the fetus, although the mechanisms of placental cross are still unknown (Ma et al., 1989; Nitsche et al., 2016; Weigand et al., 1984).

The risks to the fetus of analgesic exposure during pregnancy are still poorly studied

because of the difficulties of performing direct analyses. There is a lack of appropriate models, especially for humans and hence the majority of studies are epidemiological. There are studies that associate analgesic exposure to attention-deficit/hyperactivity disorder (ADHD) symptoms (Thompson et al., 2014), asthma (Cheelo et al., 2015; Sordillo et al., 2015), or autism (Wong et al., 2015).

1.2.2 Male reproductive disorders

Development of the male reproductive system is dependent on normal formation and function of the testis during fetal life. Failure of normal development may result in disorders that manifest in the neonatal period (cryptorchidism and hypospadias), or in adulthood (testicular cancer and poor semen quality) (Gabrielsen & Tanrikut, 2016; Bonde et al., 2017).

In humans, cryptorchidism occurs in approximately 1-4.6% of newborns, although in many cases it will resolve naturally leading to a prevalence of ~1% at 1 year of age (Radmayr et al., 2016; Sepúlveda & Egaña, 2016). Cryptorchidism is associated with an increased risk of testicular germ cell cancer (TGCC), the commonest malignancy amongst young men, and which is believed to arise from the aberrant development of a population of fetal germ cells (gonocytes), during fetal life (R. T. Mitchell et al., 2014). A history of cryptorchidism is also associated with impaired spermatogenesis in adulthood, resulting in a 30-60% risk of infertility (Niedzielski et al., 2016). Hypospadias is also a relatively common disorder occurring in approximately 0.2-0.6% of male newborns (Springer et al., 2016). The term Testicular Dysgenesis Syndrome (TDS) is frequently used to describe the association of these disorders as a result of events that occur during fetal life and their relationship with deficient androgen production or action (Skakkebaek 2001). Indeed, a critical period from embryonic day (e) 15.5 – e18.5, known as the ‘masculinization programming window’ (MPW), has been described in fetal rats during which reduction in testosterone production or androgen action leads to the subsequent development of

cryptorchidism and hypospadias (Welsh et al., 2008).

These common male reproductive disorders have a high and/or increasing incidence over recent decades, indicating that in addition to genetic abnormalities, environmental factors such as lifestyle, diet and chemical (including pharmaceutical) exposures are likely to play a role in their development (Gabrielsen & Tanrikut, 2016; Skakkebæk et al., 2016). Recently, there has been an increasing literature on the potential role of in utero exposure to commonly used analgesics, including paracetamol and NSAIDS (e.g. ibuprofen or aspirin), and their relationship to male reproductive development and its disorders. This is reviewed in detail below.

1.2.3 Analgesic exposure in pregnancy and the development of male reproductive disorders

It is not ethical to test the effects of analgesics on pregnant women directly and hence the evidence for associations between analgesic use during pregnancy and the development of male reproductive disorders derives from a combination of epidemiological and experimental studies conducted over the past two decades, but mainly in the last 3 years.

1.2.4 Association between analgesic use and the occurrence of cryptorchidism

A significant association between the overall use of mild analgesics during pregnancy and subsequent cryptorchidism in the offspring has been demonstrated in four studies with adjusted ORs of 1.33 (1.00-1.77) (Jensen et al., 2010), 1.93 (CI 1.03-3.62) (Berkowitz & Lapinski, 1996), 2.04 (CI 1.15 – 3.62) (Snijder et al., 2012) and 2.30 (CI 1.12–4.73) (Kristensen et al., 2011). However, at least one study did not find a significant association (OR 1.1; CI 0.31–3.6) (2011; Philippat et al., 2011) (Table 1). In Jensen et al, significance was only found when the whole pregnancy was taken into account, but no association was find if the exposure was only considered during 1st or 2nd trimester. In two of the studies that demonstrated an association this only

reached statistical significance for use of analgesics during the second trimester with no significant association for exposure during the first trimester (Kristensen et al., 2011; Snijder et al., 2012). Interestingly, Kristensen et al describe data on two separate Scandinavian populations. The significant associations were restricted to the Danish cohort, whilst in the Finnish cohort there were no statistically significant associations (Kristensen et al., 2011). Differences between these cohorts may relate to variations in methodology, underlying prevalence of cryptorchidism or study power. Duration of exposure may also be a factor with a significant association between prolonged (>2 weeks) use of mild analgesics during pregnancy (includes first and second trimester) and cryptorchidism (OR 2.47; CI 1.02–5.96) (Kristensen et al., 2011). In addition to the overall effects of analgesics, several of these studies have also investigated the effects of specific agents on the development of cryptorchidism.

1.2.5 Paracetamol exposure and risk of cryptorchidism

Three studies investigating associations between paracetamol use during pregnancy and cryptorchidism in the offspring have reported adjusted OR above 1.0 (Jensen et al., 2010; Kristensen et al., 2011; Snijder et al., 2012); however, only the last two found this association to be statistically significant. In one of these studies, exposure during the second trimester was significantly associated with cryptorchidism (OR 1.89; CI 1.01–3.51), similar to their results for mild analgesics overall (Snijder et al., 2012).Timing of exposure in terms of trimesters is likely to be important based on the evidence for the MPW in rodents which has been postulated to occur between 8-14 weeks in humans (van den Driesche et al., 2017). The study by Jensen et al also analysed their data from 8-14 weeks and demonstrated an HR of 1.14 (0.97 – 1.34), which was significant for a duration of exposure >4 weeks OR 1.38 (1.05 – 1.83) (Jensen et al., 2010). A second study also demonstrated a significant association OR 2.78 (1.13–6.84) following prolonged (>2 weeks) exposure to paracetamol, similar to that described for mild analgesics in general (Kristensen et al., 2011). Again, this association was restricted to the Danish cohort and was not demonstrated in the

Finnish cohort.

1.2.6 NSAID exposure and risk of cryptorchidism

The association between cryptorchidism and exposure to NSAIDS, such as ibuprofen and aspirin, has also been investigated (Table 1) (Jensen et al., 2010; Kristensen et al., 2011; Snijder et al., 2012). No significant associations were demonstrated for overall use of ibuprofen or aspirin during pregnancy(Jensen et al., 2010; Kristensen et al., 2011). However, Kristensen et al demonstrated a significant increase in risk of cryptorchidism following exposure to ibuprofen (OR 4.59; 1.10-19.0) and aspirin (OR of 3.76; 1.15-12.3) during the second trimester(Kristensen et al., 2011). A similar significant association between exposure specifically during the second trimester has also been demonstrated; however, this related to 'other' analgesics (i.e. analgesics excluding paracetamol) (Snijder et al., 2012).

These results may indicate the importance of simultaneous use of more than one analgesic during pregnancy. This has been investigated in two studies (Jensen et al., 2010; Kristensen et al., 2011). The use of 2 or more agents was associated with a significant increase in the risk of cryptorchidism (OR 7.72; CI 2.09–28.6) in one study (Kristensen et al., 2011), whilst a second study did not demonstrate a significant association (HR 1.07; CI 0.82–1.40) (Jensen et al., 2010).

1.2.7 Association between analgesic exposure and risk of hypospadias

The majority of studies investigating potential analgesic use during pregnancy and the incidence of hypospadias in the offspring have not shown significant associations (Table 1.1) (Hernandez et al., 2012; Slone et al., 1976; Snijder et al., 2012). In one study which assessed analgesic exposure from one month prior until 4 months after conception, a significantly increased risk of hypospadias has been described for ibuprofen (OR 1.20; (CI 1.00–1.30) whilst no such association was reported for paracetamol or aspirin exposure (Lind et al., 2013). Another study reported a

significant association between the use of aspirin and hypospadias (OR 3.5; CI 1.4 to 8.8) (Correy et al., 1991).

Table 1.1: Association between analgesic exposure during pregnancy and male reproductive disorders

					Analgesics	Paracetamol	Ibuprofen	Aspirin
Cryptorchidism	**Study Period**	**Cohort size**	**Publication**	**Gestational Period**	Adjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted OR (95% CI)
	1996-2002	47,400	Jensen et al., 2010	Pregnancy#	n/a	**1.33 (1.00–1.77)**	0.88 (0.64–1.19)	1.18 (0.93–1.49)
				1st trimester	n/a	0.94 (0.75 – 1.17)	n/a	n/a
				2nd trimester	n/a	1.17 (0.89 –1.54)	n/a	n/a
	2002-2006	3,184	Snijder et al., 2012	Pregnancy	n/a	n/a	n/a	n/a
				1st trimester	0.94 (0.36–2.46)	1.38 (0.52–3.64)	n/a	n/a
				2nd trimester	**2.12 (1.17–3.83)**	**1.89 (1.01–3.51)**	**8.93 (1.84–43.24)***	
	1997-2001	491	Kristensen et al., 2011 (Dannish cohort)	Pregnancy#	1.43 (0.73–2.79)	1.337 (0.70–2.55)	1.82 (0.50–6.61)	2.22 (0.86–5.76)
				1st trimester	1.48 (0.66–3.34)	1.61 (0.66–3.90)	n/a	**5.60 (1.83–17.1)**
				2nd trimester	**2.30 (1.12–4.73)**	1.97 (0.94–4.12)	**4.59 (1.10–19.0)**	**3.76 (1.15–12.3)**
	1997-1999	1463	Kristensen et al., 2011 (Finish cohort)	Pregnancy#	0.74 (0.35–1.57)	n/a	n/a	n/a
				1st trimester	0.77 (0.26–2.27)	n/a	n/a	n/a
				2nd trimester	1.21 (0.53–2.76)	n/a	n/a	n/a
	2003-2006	903	Philippat et al., 2011	Pregnancy#	1.10 (0.31–3.6)	n/a	n/a	n/a
	1987-1990	6,699	Berkowitz and Lapinski. 1996	Pregnancy	**1.93 (1.03-3.62)**	n/a	n/a	n/a
Hypospadias	2002-2006	3,184	Snijder et al., 2012	1st trimester	2.05 (0.64–6.58)	2.24 (0.60–8.32)	1.65 (0.21–13.08)*	
				2nd trimester	0.53 (0.12–2.34)	0.54 (0.12–2.41)	n/a	n/a
	1997-2007	5851	Lind et al., 2013	1st trimester	n/a	1.00 (0.80–1.10)	**1.20 (1.00–1.30)**	1.30 (0.90–1.70)
	1982-1989	56,037	Correy et al., 1991	1st trimester	n/a	n/a	n/a	**3.50 (1.40–8.80)**
	1959-1965	50,282	Slone et al., 1976	1st trimester	n/a	n/a	n/a	no association
	1997-2004	14,915	Hernandez et al., 2012	1st trimester	n/a	n/a	no association	

*applies to 'other' painkillers including NSAIDS. # refers to exposure during 1st and 2nd trimester of pregnancy.

1.2.8 Analgesic exposure and anogenital distance

Cryptorchidism and hypospadias have been linked with a reduction in androgen production or action during fetal life (Welsh et al., 2008). Anogenital distance (AGD) has been shown to be a reliable and robust indirect measure of fetal androgen exposure during the MPW in rodents (van den Driesche et al., 2017) and a reduced AGD in males has been associated with cryptorchidism and hypospadias in humans (Hsieh et al., 2014; Thankamony et al., 2014). A recent study by Lind et al investigated 1027 mother-child pairs to determine the association between analgesic exposure in mothers and AGD in the offspring at 3 months of age (Lind et al., 2017). Women were recruited from 10-27 weeks of gestation and analgesic use was assessed by questionnaire. No association was found between exposure to paracetamol or NSAID and AGD; however, exposure to a combination of paracetamol and 'other' analgesics (including NSAIDS) was associated with a reduced AGD_{AS} (ano-scrotal AGD; 32.3 v 36.2mm; p=0.03). No such association was demonstrated for AGD_{AP} (ano-penile AGD) which may relate to technical issues in conducting these measurements. In addition, this group included relatively small numbers (n=20). Further investigation of the association between AGD and in-utero exposure to analgesics would seem warranted.

Taken together, the epidemiological evidence indicates that there may be an association between in-utero exposure to analgesics, particularly during the second trimester, and cryptorchidism. The evidence for such an association with hypospadias is less convincing, but there is little data and this malformation is far less common than cryptorchidism and mild cases are generally under-diagnosed. There are a number of limitations to these studies relating to obtaining accurate information regarding the dosage, timing and duration of paracetamol/other analgesic exposure. This is particularly the case for retrospective studies. Some of the studies were performed using a case-control design involving the use of retrospective questionnaires (Berkowitz & Lapinski, 1996; Snijder et al., 2012), whilst others were

done in prospective birth cohort studies (Jensen et al., 2010; Kristensen et al., 2011).

1.2.9 Effect of analgesics on Leydig cell function in the fetal testis

Testicular descent requires the action of two hormones produced by fetal Leydig cells, namely testosterone and Insulin-like growth factor 3 (Insl3) (Hughes & Acerini, 2008). Given the reported association between analgesic exposure and cryptorchidism a number of studies have investigated the effect of exposure to various analgesics on Leydig cell function in the fetal testis. This includes in-vivo, ex-vivo and in-vitro model systems of paracetamol (Table 1.2) and NSAID (Table 1.3) exposure using rodent and human tissues.

1.2.10 Analgesics and testosterone production

Testosterone production can be measured directly (e.g. serum or intratesticular) or can be determined indirectly by measuring AGD or androgen-dependent tissue weights. Paracetamol exposure has been linked to a reduction in fetal testicular testosterone production in several studies (Kristensen et al., 2011; 2012; van den Driesche et al., 2015) (Table 1.2). In-vivo exposure of fetal rats to paracetamol (350mg/kg/d) during the MPW has been shown to significantly decrease AGD by up to 10% in late fetal life (Kristensen et al., 2011; van den Driesche et al., 2015), whilst another study only demonstrated a significant effect on AGD (15% reduction) at 10 weeks postnatally with no effect at 4,6 or 8 weeks (Holm et al., 2015). In one of these studies there was an associated significant 40% reduction in intratesticular testosterone and a significant reduction in mRNA expression of two key steroidogenic enzymes (CYP17a1 and CYP11a1) indicating a potential mechanism for the effect on steroidogenesis (van den Driesche et al., 2015). Another study in mice did not demonstrate an effect of exposure during this time-window on AGD at birth, although there was a significant effect of exposure to a chemical ‘mixture’ that included paracetamol (Axelstad et al., 2014). In-vitro studies in rats have also demonstrated effects of exposure during the MPW on testosterone production.

Exposure of e14.5 rat fetal testes to 1μM paracetamol for 48 hours resulted in a 15-50% reduction in testosterone production, with significant reductions described across a range of paracetamol doses (0.5-10μM) at 72 hours. A similar approach using in-vitro culture of human fetal testis tissue (8-12 weeks gestation) did not demonstrate any effect on testosterone production following exposure to 10μM paracetamol for 24, 48 or 72 hours (Mazaud-Guittot et al., 2013). One study has investigated the effect of exposure to a therapeutic regimen (3x daily doses of 20mg/kg giving a total exposure of 60mg/kg/d) of paracetamol on human fetal testis (14-20 weeks gestation) xenografts. In this study, a 7 day exposure to paracetamol resulted in a significant reduction in seminal vesicle weight (18% reduction) and serum testosterone (45% reduction) in the castrate nude mice hosts compared to vehicle controls, whilst there was no effect following a shorter (1 day) exposure (van den Driesche et al., 2015). The differences between the results of the two studies using human fetal testis tissue may relate to the different gestational ages or alternatively may reflect differences in the model systems used.

Table 1.2: Leydig cells function and GC/Fertility after paracetamol exposure

	Species	Model	Duration (days)	Dose*	Age (start of treatment)	Result	Publication
Testosterone	Rat	In-vivo	7	350	GD13	↔	Kristensen et al., 2011
		In-vivo	3	350	e13.5	↓ (~40%)#	van den Driesche et al., 2016
		In-vitro	2	1 µM	e14.5	↓ (~50%)	Kristensen et al., 2011
		In-vitro	2	1 µM	e14.5	↓ (~25%)	Kristensen et al., 2012
	Human	In-vitro	1	10 µM	8-12 GW	↔	Mazaud-Guittot et al., 2013
		Xenograft	7	60	14-20 GW	↓ (~50%)	van den Driesche et al., 2016
AGD	Rat	In-vivo	7	150	GD13	↓ (~10%)	Kristensen et al., 2011
		In-vivo	6	350	GD13	↔	Axelstad et al., 2015
		In-vivo	3	350	e13.5	↓ (~10%)ᵀ	van den Driesche et al., 2016
	Mouse	In-vivo	13	150	GD7	↓ (~15%)	Holm et al., 2015
INSL3	Rat	In-vivo	3	350	e13.5	↔	van den Driesche et al., 2016
		In-vitro	3	100 µM	e14.5	↔	Kristensen et al., 2012
	Human	In-vitro	3	10 µM	10-12 GW	↓ (~40%)	Mazaud-Guittot et al., 2013

If significant, the result shown is always the mínimum dose and duration used. If not significant, the highest dose and duration is shown. GW – gestational weeks. GD – gestational day. (anogenital index), calculated by dividing the GD by the cube root of body weight.

Table 1.3: Leydig cell function and GC/Fertility after NSAIDS exposure.

	Drug	Species	Model	Duration (days)	Dose*	Age (start of treatment)	Result	Publication
Testsosterone	Aspirin	Rat	In-vivo	7	200	GD13	↓	Kristensen et al., 2011
			In-vitro	1	10 µM	e14.5	↓ (~70%)	Kristensen et al., 2011,2012
		Human	In-vitro	3	100 µM	8-10 GW	↑	Mazaud-Guittot et al., 2013
			In-vitro	3	1 µM	10-12 GW	↔	Mazaud-Guittot et al., 2013
	Indomethacin	Rat	In vivo	3	0.8	e15.5	↔	Dean et al., 2013
			In-vitro	1	10 µM	e14.5	↓ (~30%)	Kristensen et al., 2012
		Human	In-vitro	2	10 µM	8-12 GW	↑ (~20%)	Mazaud-Guittot et al., 2013
AGD	Aspirin	Rat	In-vivo	7	250	GD13	↔	Kristensen et al., 2011
			In-vivo	3	150	GD11	↓ (~20%)	Gupta and Goldman. 1986
	Indomethacin		In-vivo	3	1	GD11	↓ (~20%)	Gupta and Goldman. 1986
			In-vivo	3	0.8	e15.5	↔	Dean et al., 2013
Insl3	Aspirin	Human	In-vitro	3	10 µM	8-12 GW	↔	Mazaud-Guittot et al., 2013
	Indomethacin		In-vitro	3	10 µM	8-12 GW	↔	Mazaud-Guittot et al., 2013

If significant, the result shown is always the minimum dose and duration used. If not significant, the highest dose and duration is shown. GW – gestational weeks. GD – gestational day. e – embryonic day.

*Doses are given by mg/kg/day unless otherwise stated

Many of the approaches used to investigate effects of paracetamol exposure have also been performed using NSAIDs. In-vivo studies in pregnant mice have demonstrated a significant reduction in AGD (20% reduction) in males exposed in utero to aspirin (150mg/kg/day) or indomethacin (1mg/kg/day) from GD11-14 (Gupta & Goldman, 1986), whilst no reduction in AGD was found in similar studies involving exposure during the MPW in rats (Dean et al., 2013; Kristensen et al., 2011), although in one of these studies testosterone production was reduced in the case of aspirin exposure (Kristensen et al., 2011) but not for indomethacin (A. Dean et al., 2013). However, in-vitro rat fetal testis studies have demonstrated a reduction in testosterone production following 24 hours exposure to aspirin (70% reduction; 10μM) or indomethacin (30% reduction; 10μM) from e14.5 (Kristensen et al., 2011; 2012). These findings contrast with studies using in-vitro culture of human testis tissue (8-12 GW), where exposure to indomethacin for 48 hours resulted in a significant increase in testosterone (20%; 10μM). A similar increase was demonstrated for aspirin exposure, although this was restricted to 8-10GW, with no effect at 10-12GW (Mazaud-Guittot et al., 2013). The reason for the discrepancy between the effect of NSAID exposure on testosterone production in the fetal rat and human testis is unclear and may relate to the model systems or to genuine species differences; however, this clearly illustrates potential limitations of extrapolating effects in rodent model systems directly to the human.

1.2.11 Analgesic exposure and Insl3 production

INSL3 is responsible for outgrowth of the gubernaculum testis and the first (intra-abdominal) phase of testicular descent in rodents, and mutations in the *Insl3* gene are associated with the development of cryptorchidism in mice (Nef & Parada, 1999). Given the association between analgesic exposure and cryptorchidism in humans some studies have investigated a potential role for analgesics on Insl3 production (van den Driesche et al., 2015). Paracetamol exposure (350mg/kg/d) did not result in a change in Insl3 mRNA in rat fetal testes following in-utero exposure from e13.5-

e16.5 (van den Driesche et al., 2015). This was also the case for Insl3 measured in the media following in-vitro culture of rat fetal testes (e14.5) for 72 hours. However, in human fetal tests (8-12GW) cultures, paracetamol (10μM; 72 hours) exposure resulted in a significant reduction in INSL3 production, whilst no effect was observed following exposure to the same concentrations of aspirin or indomethacin (Mazaud-Guittot et al., 2013).

1.2.12 Effect of analgesics on prostaglandins

Prostaglandins (PG) have been proposed to play a role in mediating the effects of paracetamol exposure on Leydig cell function. One study involving culture of e14.5 fetal rat testes showed a significant decrease in prostaglandin D2 (PGD2) after 24h exposure to 1μM paracetamol (Kristensen et al., 2011). However, there were no significant reductions across a range of doses (1-100μM) after culture for 24, 48 and 72h in a subsequent study by the same authors (Kristensen et al., 2012). In-vitro exposure of human fetal testis tissue (7-12 weeks) to paracetamol (10μM) for 72h did not reduce prostaglandin D2 (PGD_2) production, but it did significantly reduce prostaglandin E2 ($PGE_{2)}$ (Mazaud-Guittot et al., 2013).

For NSAIDS, the effect of exposure on production in the fetal testis appears to depend on the specific agent. A dose dependent reduction in PGD2 production after exposure to 10μM aspirin was demonstrated in culture of e14.5 fetal rat testes after 48 and 72h (Kristensen et al., 2011); however this was not confirmed in a subsequent study, with non-significant reductions only occurring after exposure to 100 μM aspirin (Kristensen et al., 2012). Similarly, for human fetal testis cultures (7-12 weeks), no effect on PGD_2 was observed following aspirin (10μM) exposure. However, similar to results for paracetamol there was a significant reduction in PGE_2 production following aspirin exposure (Mazaud-Guittot et al., 2013). For indomethacin, there was a reduction in PGD2 following in-vivo and in-vitro exposure of the fetal rat testis during the MPW (Dean et al., 2013; Kristensen et al., 2011),

which again was not demonstrated in human fetal testis cultures (Mazaud-Guittot et al., 2013). As with paracetamol and aspirin, exposure to indomethacin did result in a significant reduction in PGE_2 production, albeit this was restricted to 7-10 weeks of gestation. Moreover, studies in rats showed that in-utero exposure to indomethacin and paracetamol were able to reduce PGE_2 content in the fetal ovary, although this decrease was not significant for paracetamol ($p=0.06$) (Dean et al., 2016). Taken together, the present data relating to the effect of analgesics on PGs appear to indicate variable effects dependent, at least in part, on the specific agent and the model species used.

1.2.13 Effect of analgesics on germ cell development and fertility

Recent studies have begun to focus on the potential for analgesics to affect germ cell development and fertility including inter-generational effects of analgesic exposure (Dean et al., 2016; Holm et al., 2016). Dean et al investigated the effects of exposure of pregnant rats to 350mg/kg/day paracetamol or 0.8mg/kg/day indomethacin, during a period of gestation that includes the MPW. The pups exposed to indomethacin (male and female) showed ~50% decrease in GC number and a decrease in gonadal weight at e21.5 (Dean et al., 2016). For females this resulted in adulthood in reduced fertility, as indicated by a reduced number of pups per litter, whilst in males no effect on adult fertility was seen. For paracetamol exposure in utero, there was a similar effect on females with a significant reduction in germ cell number, gonadal weight and pups per litter (Dean et al., 2016). A similar effect on fertility has also been described for female mice exposed to paracetamol in-utero (Holm et al., 2016). However, for males, despite an overall reduction in germ cell number and gonadal weight at e21.5, there was no significant effect on fertility in adulthood (Dean et al., 2016). Further investigation of the reduced germ cell numbers in males revealed a premature differentiation of gonocytes, as evidenced by a reduction in octamer-binding transcription factor 4 (OCT4) expression, following exposure to both paracetamol and indomethacin. The premature differentiation of

this proliferative population of germ cells (i.e. gonocytes) is likely to account for the reduced germ cell number at e21.5; however, the reduction in GC number was compensated for by early puberty (Dean et al., 2016). Another study involving paracetamol (50mg/kg/d) exposure in mice (e7-e13.5) showed no effect on male germ cells at e13.5 or on germ cells or testicular weight in adulthood (Holm et al., 2015). These differences between findings in terms of the gonocyte population in fetal life may relate to differences between species, paracetamol dose or timing of exposure.

One of the most interesting aspects of the recent studies on analgesic exposure is the demonstration of effects on the F2 generation of rats, which derived from matings of the F1 exposed to paracetamol in-utero and wild-type. The F2 females exhibited a significant reduction in ovarian weight and in primordial follicle number at pnd25. Remarkably, these effects were seen independent of whether the F1 parent was male or female, raising the intriguing possibility that this may be as a result of epigenetic modification of the germline in both sexes (Dean et al., 2016).

1.2.14 Summary

Over the past 5 years several studies have investigated the potential effect of exposure to analgesics (paracetamol and NSAIDS) on the development of male reproductive disorders. Epidemiological evidence exists for associations between exposures to several analgesics and the development of cryptorchidism in boys. Experimental studies in rodents have also demonstrated effects of exposures to analgesics (particularly paracetamol) during fetal life on Leydig cell function (including testosterone production) and on GCs. Recently, in-vitro and ex-vivo (xenograft) studies using human fetal testicular tissue have been conducted which support the concept that analgesic exposure may interfere with Leydig cell function in the human fetal testis. However, differences remain between the findings of these studies that are likely to reflect variations in species, model system, dosing schedule

and timing of exposure. Further work is required to determine the potential risk that analgesics may pose to human reproductive health at human-relevant exposures. Whilst the current evidence does not provide a definitive answer to this question, avoiding pain or pyrexia is important for fetal health.

1.3 Gonad development

Pregnancy lasts 40 weeks in humans, whilst in rats, pregnancy lasts for 23 days and 20 days in the mouse. In this short period, the different tissues and organs of the body have to be developed. Every tissue has specific cell types with different gene expression (i.e. different proteome), although all of them originate from an initial totipotent cell. A succession of asymmetric divisions is necessary for all the different cell types to be formed, driven by cell/tissue-specific gene expression differences. The gonads, especially the testis, are known to express more unique genes than any other organ in the body (Djureinovic et al., 2014). The gonads contain somatic cells and germ cells (GCs). The latter are the reservoir of cells with reproductive potential. They are unique cells which carry the genetic (and epigenetic) information that will be inherited by future generations. The key role of the somatic cells is the support of the GCs (Hayashi et al., 2011).

1.3.1 Primordial germ cell migration

Fetal GCs are the progenitors of the adult gametes. They are regulated in a spatio-temporal manner by complex regulatory networks. The founder cells of the GC lineage are the primordial germ cells (PGC). They first appear early in embryonic development, in week 4 of human pregnancy and e11 in the mouse (Culty, 2009). Different species have different mechanisms for specification of the PGCs. For example, some invertebrates, such as Caenorhabditis *elegans* or *Drosophila Melanogaster* have the factors needed already in the maternal eggs, localized in one pole of the cell (Strome & Lehmann, 2007). In other animals, including mammals, some cells in the epiblast differentiate to become PGCs by expressing *Blimp1/Prdm1*, *PRDM14* and *Fragilis* (Hayashi et al., 2007). At this stage, epiblast cells are still pluripotent but they are getting signals that will shift them towards a somatic fate. However, Bone morphogenetic proteins (BMP), such as BMP2, BMP4 and BMP8b, signals induce the expression of the aforementioned PGC markers. Not only that, BMPs also block the expression of somatic cell markers, such as Homeotic genes

(Hox) and promote re-expression of lost markers of pluripotency, such as Sox2 (Saitou et al., 2012). A cluster of around 40 PGCs are formed and start to migrate through the developing hindgut to colonize the gonadal primordia, also known as genital ridges. The PGCs arrive to the genital ridges at the onset of the development of the future gonads and by then, they have reached a number of around 1000 in the mouse (Richardson & Lehmann, 2010; Tam & Snow, 1981). The PGCs will be in their final location at approximately e10.5 in the mouse and 6 weeks in the human (Saitou et al., 2012). During migration, PGCs undergo important modifications, such as global DNA demethylation, erasure of imprinted genes and global G2-arrest (Hajkova et al., 2002; Richardson & Lehmann, 2010; Saitou et al., 2012). These modifications will define their role as GCs and prepare them for meiosis. (Figure 1.1).

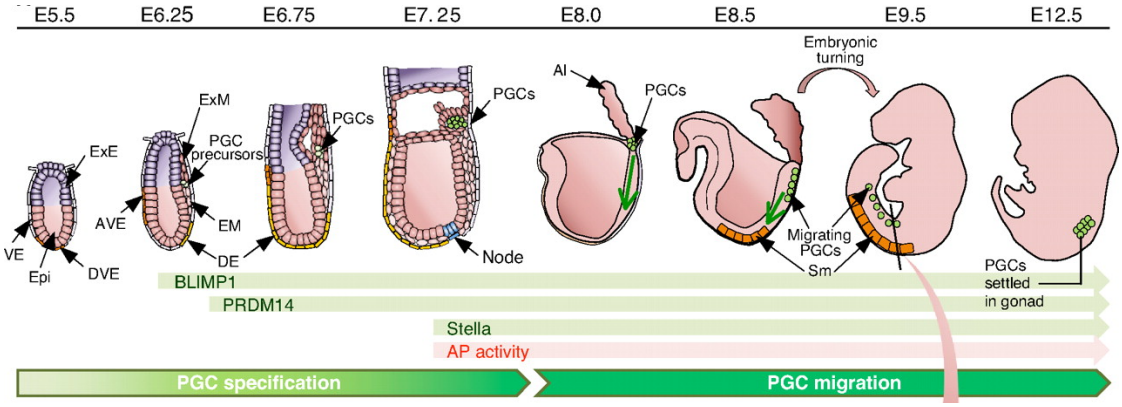


Figure 1.1: Specification and migration of mouse primordial germ cells. GC specification and migration in developing mouse embryos. At e6.25, PGC precursors appear. PGCs (in green), proliferate from e6.25 to e12.5. Migration occurs towards the the genital ridge (direction of the green arrow. The timing of expression of key genes (*Blimp1, Prdm14* and Stella) and alkaline-phosphastase activity is shown below. (Adapted from: Saitou et al., 2012).

1.3.2 The bipotential gonad

During the first stages of gonad development, both males and females undergo the same changes and modifications. During early embryo development, the urogenital system is formed, comprising three different regions: pronephros, mesonephros and metanophos. While the kidneys will develop from the metanephros, the gonads

appear from the medial side of the mesonephros (Byskov, 1986). The development of the gonads involves proliferation of the coelomic epithelium which overlies the mesonephroi (Merchant, 1975; Schmahl et al., 2000). The biopotential gonads are characterized by the expression of Wilms tumour protein 1 (Wt1) and steroidogenic factor 1 (Sf1) (Wilhelm & Englert, 2002).

1.3.2.1 Sex determination

Once they arrive in the genital ridge, GCs will develop differently between male and females. This different behaviour is presumed to be driven by sex differences in gonad somatic cell composition (She & Yang, 2014). In mammals, male gonad differentiation is initiated by the presence (and expression) of the Sex determining region of the Y chromosome (*Sry)* gene in somatic cells (Gubbay et al., 1990; Koopman et al., 1991; Sinclair et al., 1990). Experiments in rodents have shown that *Sry* knockout mice present with a normal female phenotype with ovaries (Gubbay et al., 1992; Hawkins et al., 1992). These experiments confirmed the idea that the basic set-up program is the female pathway. The role of *Sry* is to enhance *Sox9* expression, together with Sf1, in Sertoli cell precursors, which initiates a new expression cascade of different factors that will drive these cells to fully differentiate into Sertoli cells (Figure 1.2) (Sekido & Lovell-Badge, 2008). These cells are essential for testis development and orchestrate cord formation, sex-specific GC development, the differentiation of fetal Leydig cells, etc. (She & Yang, 2016). *Sox9* expression persists in the testis during fetal development and adult life (Kent et al., 1996). Using the same principle as in *Sry* knockout, gonads in $Sox9^{-/-}$ XY mice will develop as ovaries whilst XX gonads expressing *Sry* and/or *Sox9* will develop as testes (Koopman et al., 1991; Vidal et al., 2001). Some cases of sex reversal in human XY genotypic males can be explained by mutations in *SRY* or *SOX9* (Kiefer, 2007).

Even if expression of a single gene is enough to produce a male phenotype, the process is rather more complex than this. Becoming female may be the set-up

pathway, but it is not passive. Both female and male pathways are constantly and actively repressing each other via mutual antagonism that involves expression of numerous different genes. In the absence of *SRY* expression, as in normal females, but also in many disorders of sex development (DSDs), the female cascade of gene expression involved in ovarian differentiation is not suppressed (Kiefer, 2007; She & Yang, 2016). The discovery of the factors which are actively involved in ovarian determination was achieved thanks to the use of different animal models and the study of DSD patients. Some of these factors are ForkheadboxL2 (FOXL2), wingless type MMTV integration site family member 4 (WNT4), R-spondin 1 (RSPO1) and β-CATENIN (Elzaiat et al., 2016). The products of these genes actively inhibit *SOX9* expression at the same time as they promote Müllerian duct differentiation and oocyte development and hence, female development (figure 1.2) (Biason-Lauber & Chaboissier, 2015). FOXL2 is the earliest known marker of ovarian differentiation (Cocquet et al., 2002). FOXL2 is expressed in somatic cells that will develop as pre-granulosa cells and It is an essential transcription factor in ovarian development (Georges et al., 2014). In mice, Foxl2$^{(-/-)}$ ovaries showed activation of male sex determination genes including Sox9, with consequently partial secondary sex reversal including the development of Sertoli-like cells (Ottolenghi et al., 2005). Partial sex reversal is seen after conditional loss of Foxl2 in the adult, where granulosa and theca cells start expressing Sertoli and Leydig cell factors, consequently leading to an inappropriate (for females) increase in testosterone levels (Uhlenhaut et al., 2009). Larger mammals, such as the goat, also undergo a complete sex reversal in the absence of FOXL2. This is a well described phenotype and is known as polled intersex syndrome (Boulanger et al., 2014). However, the literature shows differences in the effects of FOXL2 mutations between other mammals and humans, in which it results in a different spectrum of disorders including premature ovarian failure, but not sex reversal (Harris et al., 2002).

In fetal gonads of mammalian females, Rspo1 regulates W*nt4*, which in turn controls

β-catenin stability and localization (Figure 1.2). In the absence of wnt4, β-catenin is ubiquitinated and degraded. When Wnt4 levels are increased by Rspo1, β-catenin is accumulated in the cytoplasm and then translocated into the nucleus where it induces the expression of different target genes after the formation of a complex with the transcription factor LEF/TCF (Clevers & Nusse, 2012). Deletion of *Wnt4* or *Rspo1* in fetal mice results in trans-differentiation of granulosa cells into Sertoli cells (Lavery et al., 2012; Nicol & Yao, 2015). Mutations in *WNT4* and *RSPO1* in humans is associated with a spectrum of disorders such as ambiguous genitalia and severe hypospadias, or renal agenesis and gonadal morphology ranging from an ovotestis to a normal testis (Jordan et al., 2001; Domenice et al., 2004; Parma et al., 2006).

As outlined above, somatic cells in the mammalian male gonads express genes that actively repress the female gene expression pathway. Thus, Dmrt1 represses *FoxL2*, *Wnt4* and *Rspo1*, at the same time as it promotes *Sox9* expression (Figure 1.2) (Matson et al., 2011). This general repression of the female differentiation by the male gonads is also supported by other downstream molecules involved in the male pathway, such as fibroblast growth factor 9 (Fgf9) and prostaglandin D2 synthetase (Pgds) (Dong et al., 2015) (Figure 1.2).

The process of gonad differentiation continues throughout the period of fetal development. Different molecular factors, including hormones, coordinate the development of the differentiated gonad. This includes the development of the reproductive ductal systems and the formation of the appropriate external genitalia as well as the rest of the secondary sexual characteristics. Modifications of any aspect of these processes can result in a variety of sexual abnormalities and DSDs.

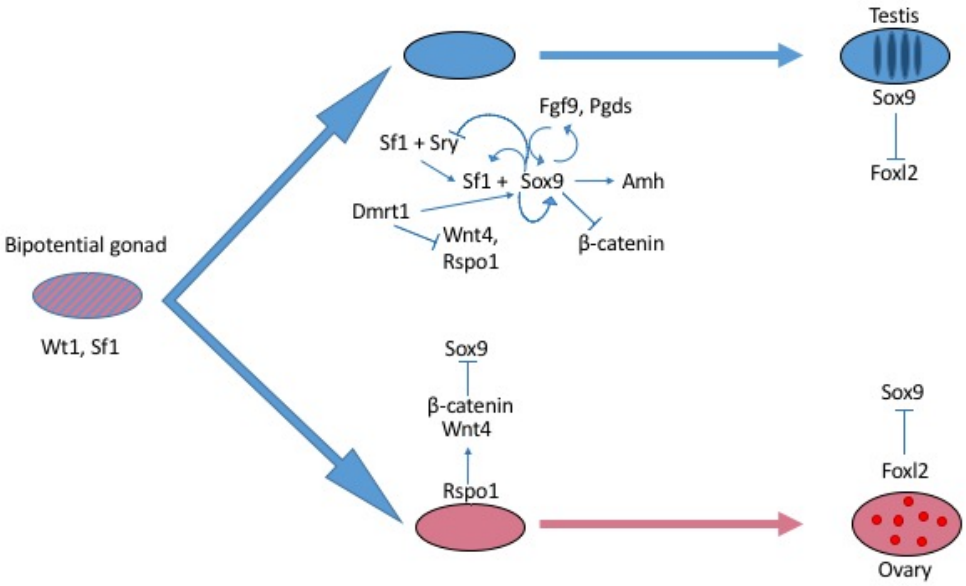


Figure 1.2: Mammalian gonad sex determination derived from mouse studies. The bipotential gonad is established by the expression of specific genes including Sf1 and Wt1. In XX gonads, Rspo-1 boosts the expression of *Wnt4* and *β-catenin*, which start to accumulate. Later in development, Foxl2 increases, helping to maintain granulosa cell differentiation by the repression of *Sox9*. As a consequence, β-catenin levels accumulate sufficiently to repress Sox9 activity. In XY supporting cell precursors, *Sry* is activated as a consequence of increasing levels of Sf1. Sry, together with Sf1, triggers *Sox9* expression. Once Sox9 levels reach a critical threshold, several positive regulatory loops are initiated. In the testis, Sox9 stimulates the testis pathway by Amh activation, but also by repression of ovarian genes, such as *Wnt4* and *Foxl2*.

1.3.3 Male reproductive system development

The majority of the research performed on the development of the male reproductive system has been derived using mice as a model, and hence the translation of our knowledge into humans must be taken with a degree of caution. Sry expression in the XY genital ridge induces various morphological changes. The cells which form the the coelomic epithelium start to proliferate, driven by Fgf9 (Schmahl et al., 2000; Schmahl & Capel, 2003), which increases the size of the gonad. These cells will differentiate into Sertoli (Karl & Capel, 1998; Schmahl et al., 2000) and fetal Leydig cells (Defalco et al., 2011). There is also the development of a sex-

specific vasculature pattern. This appears to derive from endothelial cells that have migrated from the adjacent mesonephros into the testis. The developing vascular system surrounds the somatic and germ cell aggregates, and may play a role in inducing or supporting seminiferous cord formation via the vascular endothelial growth factor (VEGF) signal (Sargent et al., 2015). The mesonephros also undergoes morphological differentiation as a response to *Sry* expression, which induces the migration of somatic cells (Capel et al., 1999). This cell migration is needed for the formation of the testis cords in male gonads (Buehr et al., 1993; Capel et al., 1999). For normal testis development in mice, the expression of *Sry* is necessary during a short period of time (6h; 11.0-11.25 dpc). When *Sry* expression in the fetal gonad was delayed using an inducible Sry transgene, it resulted in an absence of testis cords due to the failure of Sertoli cell differentiation (Hiramatsu et al., 2009). A similar phenotype is seen after inactivation of *Wt1*, presumably due to the reduced expression of *Sox9* that results (Gao et al., 2006).

Once they have differentiated, Sertoli cells start producing and secreting anti-Müllerian hormone (AMH), a member of the transforming growth factor-β (TGF-β) family. AMH, previously known as Müllerian inhibiting substance, was discovered by the original studies of Alfred Jost, resolving the disagreement existing in the scientific community to explain the mechanism of somatic sex differentiation (Jost, 1956). AMH signalling is an essential, but also sufficient, player to induce regression of the Müllerian ducts (Behringer et al., 1990; Behringer et al., 1994; Tsuji et al., 1992). Different mutations have been found in the human *AMH* gene. As a consequence, individuals with these mutations showed highly reduced AMH levels, which resulted in males with persistence of the derivatives of the Müllerian ducts (uterus and fallopian tubes) (Belville et al., 1999).

As well as undergoing regression of the Müllerian ducts, males need to differentiate the Wolffian ducts, which in humans occurs between weeks 9 and 13 of gestation.

To do so, the testis needs to produce testosterone, which is generated by the fetal Leydig cells. Androgens activate a variety of Hox genes, but also different growth factors. These factors will promote the differentiation of the Wolffian duct to produce the epididymis, vas deferens and seminal vesicles (Hannema & Hughes, 2007). Some of the most studied genes involved in the development of the Wolffian ducts includes Paired box 2 (*Pax2)* and *Lim1.* $Pax2^{(-/-)}$ mice do not develop kidneys, genital ducts or mesonephric tubules (Torres et al., 1995). Mice lacking Lim1, which has been proposed to interact with Pax2, do not have wolffian or Müllerian duct derivates (Bouchard et al., 2002; Kobayashi & Behringer, 2003).

Other essential steps in male development include differentiation of the prostate and male external genitalia. These processes are driven by dihydrotestosterone (DHT), a more potent androgen which is converted from fetal testosterone by 5-αreductase within the target tissues (Yamada et al., 2006) The prostate differentiates from the cranial region of the urogenital sinus by the expression of androgen receptors and the induction of paracrine factors secreted by the mesenchyme (Marker et al., 2003; Richter et al., 2007). In humans, the first prostatic buds appear around the 10^{th} week of gestation and at e17 in mice (Timms et al., 1994). Similar to the gonads, the external genitalia is formed as a bipotential anlagen in males and females. In males, DHT increases cellular proliferation in the genital tubercle and the subsequent developing penis internalizes the urethra by the invasion of the urorectal septum into the phallus (Cohn, 2004; Perriton et al., 2002). Alterations of the normal male external development pathway, such as from deficiency of DHT production, result in a wide range of DSDs, including some minor physiological defects or fully external sex reversal (Flück & Pandey, 2014; Van Batavia & Kolon, 2016).

One of the differences in male fetal development between rodents and human is the implication of the fetal adrenals and its androgens. While in rodents the main source of testosterone comes from fetal gonads, in humans there is a second pathway of

testosterone production involving fetal adrenals. This second pathway is also known as the “Alternative backdoor pathway”, although it is not clear how important is for human development (Flück et al., 2011). However, different mutations in human produces fetal phenotypes that can only explain by the act of this alternative pathway. For example, fetuses with 21-hydroxylase deficiency have high amounts of 17-hydroprogesterone, which cannot be converted to testosterone by the gonads. Yet, female fetuses with this deficiency suffer severely virilised during pregnancy, suggesting a conversion of 17-hydroprogesterone into testosterone by an alternative pathway (Auchus et al., 1998; Speiser et al., 2010).

1.3.4 Female reproductive system development

In the absence of the Y-chromosome (i.e no *Sry/Sox9* expression) the genital ridge will proceed towards an ovarian fate. As a gonad, the main functions of the ovary are the production of hormones and the generation of oocytes that will able to be fertilized in adulthood. In the testis, the main functional unit is the testis cord and it is formed in fetal life as stated before. However, in the ovary the functional unit is the ovarian follicle. The timing of ovarian follicle differentiation is different between species. It only starts after birth in rodents (Smith et al., 2014), while in humans it starts during pregnancy, after week 16 of gestation (Grive & Freiman, 2015) (Figure 1.3).

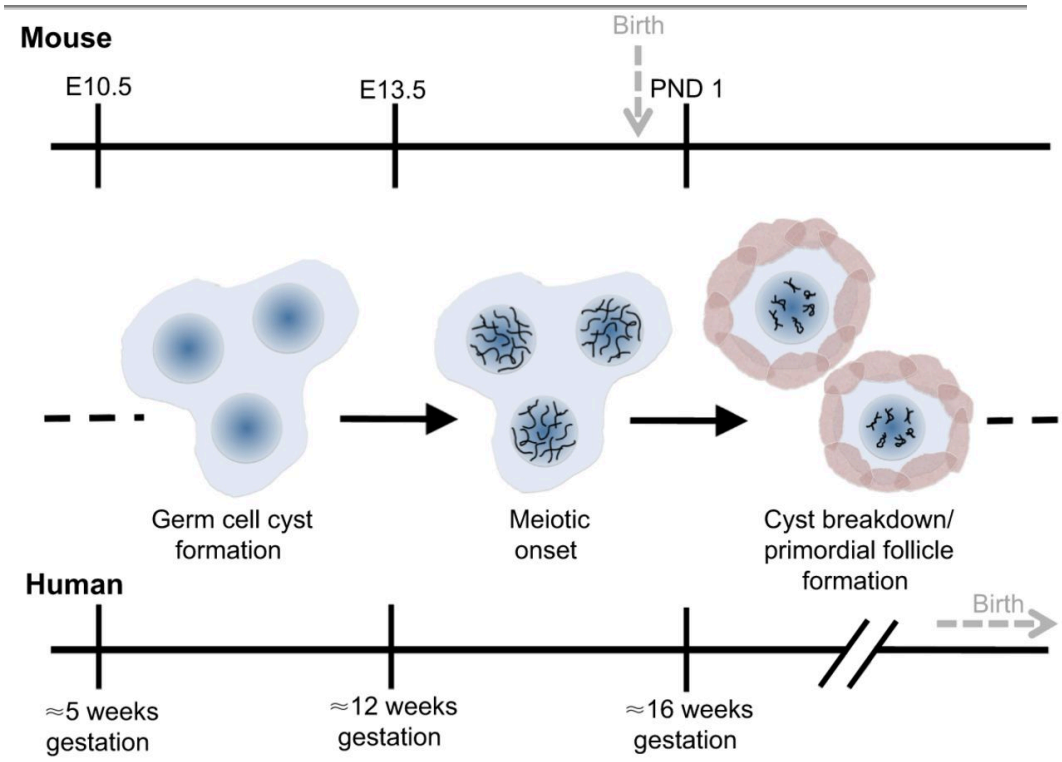


Figure 1.3: **Comparative timelines of primordial follicle formation in mouse and humans.** Timeline comparing cyst formation, meiotic onset and primordial follicle formation between mice and humans. PGCs colonize the genital ridge at about e10.5 in mice and 5 gestational weeks in humans. Cyst formation occurs between e10.5 and e13.5 in mice and between 10 and 12 weeks is human, what is just followed by meiotic onset. Cysts break around birth in mice, while in humans, it begins during second trimester (around 16 weeks) (Obtained from Grive et al., 2015).

While the testis has a fast growth rate and morphological differentiation, ovarian development has a slower pace and appears rather inert for several days in mice, although the ovary expresses female specific genes after e11.5 (Bowles & Bullejos, 2000; Nef et al., 2005; Yao et al., 2004). One of the first distinctive morphological modifications in the developing ovary is the formation of germ cell nests in the outer cortex (Wilhelm et al., 2007). Thus GCs become surrounded by somatic cells, forming structures called nests (McLaren, 2003; Pepling, 2006). These somatic cells, the pre-granulosa cells, are the equivalent cell type to Sertoli cells in the male. Inside the nests, GC multiply for several days by mitosis with incomplete cytokinesis, so that the GCs remain connected via cytoplasmic bridges (Rajah et al., 1992). The process of mitosis is synchronized in mice within the same nest, meaning that all GC within the

same nest are in the same stage of development (Pepling & Spradling, 1998). However, in humans, GC division is unsynchronised and cells within the same nest can be found in different stages of development (Stoop et al., 2005). The role of the nests is well conserved throughout evolution. It appears not only in frogs (Kloc et al., 2004), but also in invertebrates such as the grasshopper and *Drosophila melanogaster* (de Cuevas et al., 1997). The nests eventually break down to form the primordial follicles. This process occurs during the second trimester of gestation in humans, while in mice it occurs just after birth (Bayne et al., 2004; Tingen et al., 2009).

As outlined earlier, AMH secretion causes degeneration of the Müllerian ducts in males. As AMH is not produced by the fetal ovary, the Müllerian ducts persist and develop to form the fallopian tubes, uterus, cervix and upper portion of the vagina. However, the development of the female reproductive system is not passive or a default pathway. It is an active process controlled by precise and regional development involving several different factors including the *Hox* genes, which can be partially regulated by oestrogen (Du & Taylor, 2004). Abnormal modification of the pattern of expression of the *Hox* genes involved in this part of development, such as *Hoxa10* or *Hoxa11,* can lead to reproductive tract anomalies such as a T-shaped uterus, where the uterus is branched and narrowed (Goodman, 2002).

1.3.5 Germ cells

The main role of GCs is to transmit the genetic information to the next generation. However, in some species, such as in zebrafish, they are also involved in gonadogenesis and absence of GC results in testis differentiation (Slanchev et al., 2005). In mammals, GCs are not needed for normal fetal sex differentiation, but they can be involved in normal gonad development, at least in females (Handel & Eppig, 1979; Maatouk et al., 2012). Depletion of rodent GCs can cause disruption in ovarian structure and normal folliculogenesis after birth (Guigon et al., 2005; Mazaud-Guittot

et al., 2006). On the contrary, GC depletion in male fetal rodents results in normal testis development (Handel & Eppig, 1979).

GCs must ultimately go through meiosis to be able to become oocytes/sperm. Once GCs arrive in the genital ridge, they undergo a transition in order to be prepared for meiosis. This transition is driven by the transcription of specific genes within the somatic cells, which give support to the process. Different proteins have a role in this transition and one of the most studied is Deleted in Azoospermia Like (Dazl), which starts to be expressed in XX and XY GCs before their sexual differentiation (Seligman & Page, 1998). In the absence of Dazl, PGCs migrate to the gonad but fail to follow normal development along the male or female pathway, leading to failure of oogenesis and spermatogenesis (Gill et al., 2011).

The environment created by the somatic and germ cells in fetal gonads, which is different in male and female, will determine the timing of meiosis onset. Female GCs start the process during fetal development, such that when nest formation concludes, most of the oogonia nuclei are arrested in the diplotene stage of meiotic prophase I, but the process will not restart until ovulation (Tingen et al., 2009). In contrast, male GCs will not enter meiosis until puberty, which is 11-13 years later in humans and 20-35 days in rodents. After the mitotic expansion of oogonia, at about 13.5 dpc in mice (McLaren, 2000) and week 11-12 of gestation in humans (Gondos et al., 1986; Kerr et al., 2008), the oogonia enter meiosis as a result of somatic cell-derived signals, including retinoic acid (RA) in rodents and humans, although some aspects of the regulation process seem to differ between these species (Feng et al., 2014a; Le Bouffant et al., 2010; Spiller et al., 2012). RA is produced by the mesonephros inducing the expression of 'Stimulated by retinoic acid 8' (*Stra8*) by the germ cells (Koubova et al., 2006), which is indispensable for triggering the onset of meiosis. Different pathways are initiated as part of this complex process. One of them is Notch pathway, which is believed to be in charge of maintaining the epigenetic

state of *Stra8*. When the Notch pathway is repressed by the use of specific inhibitors, meiosis is delayed in oocytes (Feng et al., 2014b).

In males, contrary to females, meiosis does not start during fetal development. In order to stop the start of meiosis, there is an active process for preventing the meiotic entry of gonocytes (Bowles & Koopman, 2010; Guerquin et al., 2010). This process is not completely understood (especially in humans) but can involve several mechanisms, including Cyp26B1, Nanos2 or Nodal, all of which can inhibit expression of Stra8 (Bowles & Koopman, 2010; Souquet et al., 2012; Suzuki & Saga, 2008). Doublesex and mab-3 related transcription factor1 (Dmrt1) decreases RA responsiveness in spermatogonia as well as repressing Stra8 transcription (Matson et al., 2010). However, in females, Dmrt1 has the opposite effect, activating Stra8 and allowing the onset of meiosis and then disappearing from GCs by e15.5 in mice (Jorgensen et al., 2012). The POU transcription factor *Oct4* also plays a role in meiosis initiation. This factor is expressed in early mouse embryogenesis and in pluripotent stem cell lines. It is actually one of the four factors that Yamanaka used to generate induced pluripotent stem cells (iPS) in the studies that gained him the Nobel prize. After the gastrulation stage, Oct4 is only present in the germline. Many studies have shown a link between Oct4 and the start of meiosis as a molecular trigger playing a role in the commitment to meiosis. However, Oct4 is then downregulated during oogenesis and spermatogenesis (Pesce et al., 1998).

GC expression profiles have been highly studied. Shortly after their specification, PGCs express markers of pluripotency. Expression of these markers is maintained through development until the stage of fetal/infantile spermatogonia in the case of the testis and oogonia in the ovary. GCs express some genes in common with embryonic stem cells, such as *Oct3/4* and *Nanog*, but also some that are GC-specific, such as *blimp1*. The gene expression profile evolves with GC development. Human and rodent GC have similar profiles, but with some differences, such as the

expression of *SOX17* by human (and not by rodents) and the expression of *Sox2* by rodent GCs but not by human (Irie et al., 2015; Perrett et al., 2008).

1.4 Epigenetic mechanisms

Waddington proposed in 1942 the first theory about the study of the developmental processes between the genotype and the phenotype: epigenetics. He described it as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (Waddington, 1942). In a more modern perspective, an epigenetic trait is defined as a heritable change in gene expression or cellular phenotype, which does not change the DNA sequence (Deichmann, 2016). The epigenetic field has been deeply studied during the last two decades, however its role in physiology, disease and intergenerational effects are still largely unknown (Goldberg et al., 2007). Epigenetic modifications have been described across all species of animals and plants, but also in bacteria and yeasts (Capuano et al., 2014). The epigenome of a cell can vary for many different reasons including internal mechanisms related to cell development, growth, division, specification, etc. External stress, such as dietary components, temperature change or chemical components can also modify the epigenetics of a cell (Feil & Fraga, 2012). Various epigenetic modifications have been associated with autoimmune diseases (Herzig et al., 2016), type 2 diabetes (Sommese et al., 2017) and especially with different types of cancer (Verma & Srivastava, 2002).

The expression of a specific gene will depend on the accessibility of the transcription machinery to the DNA. A more accessible gene will be easier to be transcribed and so, modifications in the DNA structure and/or in the proteins interacting with the DNA can change gene expression by altering the transcription machinery access (Jin et al., 2011). Different main mechanisms of epigenetic regulation have been discovered so far, including histone modifications, cytosine methylation (DNA methylation) and non-coding RNAs, which can interfere with mRNA translation and

thus, with gene expression. Only the first two will be covered in this thesis.

1.4.1 Chromatin

1.4.1.1 Histones

Cellular DNA is organized in the nucleus with the help of different proteins which together comprise the chromatin. The principal components of the chromatin are the nucleosomes, formed by five types of histones (H1, H2A, H2B, H3 and H4), along with 147 pairs of DNA. Within the nucleosome, histones are organized in octamers, comprised of 2 copies of each histone type (except H1), which are then wrapped by the DNA, assisted by histone H1 which acts as a linker (Di Croce & Helin, 2013).

There are other types of histones involved in the dynamics of the chromatin in different cells and tissues. However, the presence of some of these variants is restricted to the germ cells and they might be related to the meiotic process in males and females, but also with DNA packaging in the case of sperm. Examples of these are the H1 variants, H1t, H1t2 or HILS2, the loss of which is related with problems in chromatin condensation (Martianov et al., 2005; Rathke et al., 2014); H2 variants H2A.X and H2A.Y, TH2A or TH2B, expressed at specific points of male GC development (Govin et al., 2007; Rathke et al., 2014) and H2A.Bbd, H2A.Z or H2A.X appear during oocyte meiosis (Wu et al., 2014); H3 variants involve H3.3A expressed in post-meiotic and H3.3B, which can be found only during meiotic prophase both in the case of spermatids (Rathke et al., 2014) and H3.1, involved in oocyte chromatin remodelling that occurs during GC development (Kawamura et al., 2012).

1.4.1.2 Histone modifications

Histones can undergo a large number of post translational modifications (PTMs) in the form of added residues, especially in their tails (Di Croce & Helin, 2013). Literature has shown, so far, eight distinct types of PTMs: Acetylation, lysine methylation, arginine methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deamination, proline isomerization, crotonylation (Table 1.4). The

most well studied are the small covalent modifications acetylation, methylation and phosphorylation (Kouzarides, 2007).

The variety of histone modifications is not only due to the number of different existing modifications, but also to the position where they can be added within the histones. Residues have been found in over 60 histone tails. An extra level of complexity is added from the fact that each residue may contain more than one modification, especially methylations and acetylations, which can be mono-, di- or tri- methylated/acetylated (Kouzarides, 2007). Differences in location and/or the residues involved may result in a different functional change, as such a system provides incredible flexibility (as well as complexity). For example, histone 3 methylation can result in gene repression when it is added to the lysine 27 of histone 3 (H3K27me3), but gene activation when it is added to lysine 4 of histone 3 (H3K4me3) (Di Croce & Helin, 2013; Smith et al., 2004).

The function of histone modifications normally falls into two categories: global chromatin environments and biological tasks. Histone PTMs can establish a global chromatin environment by, for example, differentiating euchromatin (where DNA is accessible for transcription) from heterochromatin (DNA is inaccessible and there is no transcription) (Bannister & Kouzarides, 2011). On the other hand, modifications can act in particular cells or at specific time points by unravelling the chromatin of specific genes. This can occur in a wide number of processes, such as DNA replication or chromosome condensation (Doyon et al., 2006), or a more local function, such a DNA repair (Fillingham et al., 2006). Biological tasks are more complex because they require the unravelling of DNA by recruiting the appropriate machinery, but also restoring the correct chromatin state when finished (Kouzarides, 2007).

Histone PTMs are regulated by specific enzymes. Most of these enzymes are specific for the residue and the modification. The identity of the enzymes responsible for methylation (Zhang & Reinberg, 2001), acetylation (Darieva et al., 2015),

phosphorylation (Nowak & Corces, 2004), sumoylation (Huang et al., 2016), deamination (Cuthbert et al., 2004), ubiquitination (Shilatifard, 2006) and proline isomerization (Nelson et al., 2006) have all been identified. Enzymes adding modifications normally work dynamically with other enzymes, the role of which is to remove these modifications. However, the enzymes currently identified are not able to explain all of the histone modifications that have been found (Kouzarides, 2007).

Many of the enzymes in charge of adding or removing histone modifications act within a group of proteins, such as Trithorax group (TrxG) or the Polycomb group (PcG). These examples were discovered many years ago in *Drosophila melanogaster* because of their activator/repressor roles in homeotic (Hox) genes (Lewis, 1978). A large part of the research done with these group proteins is still performed in *Drosophila melanogaster* as the main model, but yeast or mammal models have also been used.

Table 1.4. Different classes of modifications identified on histones. Adapted from (Kouzarides, 2007)

Modification	Residue modified	Functions regulated
Acetylation	**K**-ac	Transcription, repair, replication, condensation
Lysine methylation	**K**-me1, **K**-me2, **K**-me3	Transcription, repair
Arginine methylation	**R**-me1, **R**-me2a, **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, repair, condensation
Ubiquination	**K**-ub	Transcription, repair
Sumolynation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deamination	**R**>Cit	Transcription
Proline isomerisation	**P**-cis>**P**-trans	Transcription
Crotonylation	**L**-Kcr	Resistance to repressors

1.4.1.3 Polycomb group (PcG)

PcG is divided into two different polycomb repressive complexes (PRC), named PRC1 and PRC2. Both groups have different roles, but they act together in repressing genes by adding three methylations to lysine 27 of histone 3 (H3K27me3), a common marker of gene repression. In mammals, PRC2 is created by the association of three different proteins named suppressor of zeste 12 (SUZ12), ectoderm development (EED) and enhancer of zeste 2 (EZH2) (Di Croce & Helin, 2013). In some situations, EZH2 is substituted by a close homolog, EZH1 (Margueron et al., 2008). The composition of the PRC1 core is more variable as it is formed by one chromobox-domain (CBX2-CBX8), one member of the PCGF family (PCGF1-PCGF6), one member of the HPH family (HPH1-HPH3) and one of the RING1 family (RING1a and RING1b) (Cao et al., 2005; Wang et al., 2004). Some researchers have focused on the requirement of specific homologues of these proteins. Studies on different CBX KO mice have shown the importance of the different homologues for normal development. CBX7 is not essential for mouse development, while CBX2 and CBX4 knockout mice displayed postnatal lethality, and showed phenotypes such as male-to-female sex reversal and defects in skeletal development (Baumann & La Fuente, 2011; Forzati et al., 2012; Liu et al., 2013). Many PcG proteins are also found in other complexes related to histone modifications (Figure 1.4) (Di Croce & Helin, 2013).

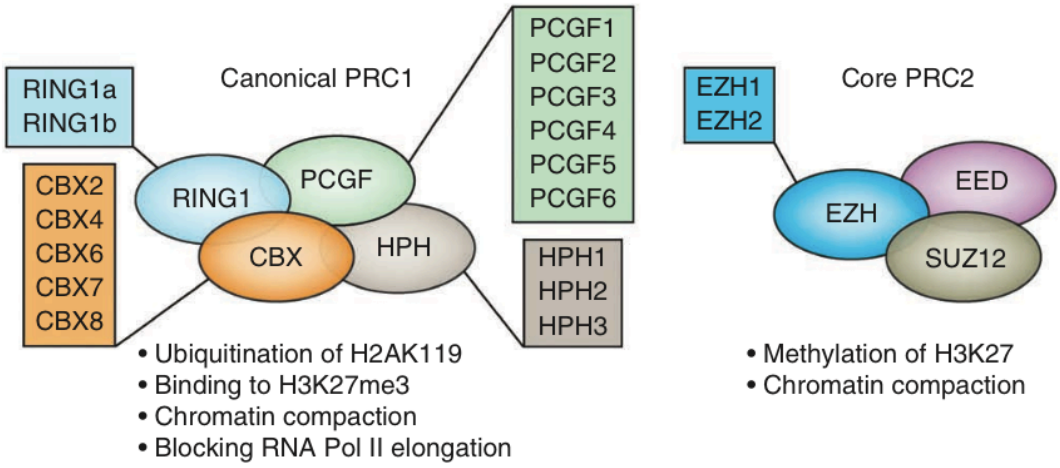


Figure 1.4: Composition and function of the main Polycomb complexes. Different Polycomb complexes can be formed by the combination of homologous proteins of the main components. (Adapted from Di Croce & Helin, 2013)

The variability in the proteins of the PRC1 creates the possibility of many different PRC1-like complexes, however, so far only 6 have been discovered (Gao et al., 2012). The role of the different PRC1 complexes is still largely unknown. Nonetheless, some publications have shown different roles according to the composition of the complexes. For example, in pluripotent ESC the genes targeted by the complex can differ depending on the proteins forming the PRC1 complex (Morey et al., 2012).

As stated earlier, the role of PrGs is to promote the repression of specific genes by the addition of H3K27me3, which generally occurs by a joint action of both PRC1 and PRC2. The PRC2 complex can be recruited to target genes thanks to sequence-specific transcription factors called polycomb response elements (PREs). In *Drosophila melanogaster* PREs exhibit dynamic interactions between different regulatory factors in a cell or tissue-specific manner (Brown & Kassis, 2013; Oktaba et al., 2008). In mammals, the process is more complex and it depends on different sequences, such as REST, PLZF/RARA or SNAIL, which are not well studied and not completely understood (Arnold et al., 2013; Boukarabila et al., 2009; Herranz et al., 2008). Other studies have reported a tight correlation between the presence of the PRC2 complex and CpG islands, showing that CG-rich regions are enough to recruit the PRC2 complex (Ku et al., 2008; Mendenhall et al., 2010). Some external proteins can also be involved in specific PRC2 recruitment. Experiments using Ten-eleven translocation 1 (Tet1) KO mice showed how this protein (traditionally studied in relation to DNA methylation) helps the silencing of Polycomb-targeted developmental regulators. Tet1 contributes and facilitates PRC2 recruitment to CPG-rich gene promoters (Wu et al., 2011). This is discussed further in section 1.4.3.

Once the PRC2 complex is recruited to a specific site, the methyltransferase EZH2 is able to add the H3K27me3. However, this is not enough for gene repression and PRC1 is needed to amplify the signal by mono-ubiquitination of H2A on lysine 119 (H2AK119ub1) by Ring1b (Wang et al., 2004). This histone modification has been

proposed to restrain RNA Polymerase II activity and to prevent the removal of the H2A-H2B dimers, which are necessary for transcription elongation (Zhou et al., 2008b). In the case of PRC1, CBX is attracted by H3K27me, which can act as a docking site (Di Croce & Helin, 2013). For these reasons, PRC1 recruitment has been tightly linked with PRC2 in a wide diversity of publications. However, this view has been recently challenged following some experiments using chromatin inmmunoprecipitation (ChIP) in *Drosophila melanogaster*, suggesting that PRC1 and PRC2 can interact independently of histone modifications (Kahn et al., 2016). Adding extra complexity, some forms of PRC1 do not contain CBX (non-canonical forms) and they are not able to recognize H3K27me3. Instead, these forms directly attach to some DNA-binding sequences, such as Fbxl10 and E2F6, although their role is still related to H3k27me3 (Ogawa et al., 2002; Wu et al., 2013)

1.4.1.4 Trithorax group

TrxG are also involved in general transcription processes via the deposition of three methylations on lysine 4 of histone H3 (H3K4me3), a common marker of enhanced gene expression (Smith et al., 2004). In a similar way to the PRC complexes, TrxG are also formed by different subunits acting together. The proteins comprising TrxG can be divided into three different groups: metyltransferases, such as ASH1 and TRX which mostly target H3K4 (Smith et al., 2004), ATP- dependent chromatin remodelling factors (able to read histone methylation marks) and DNA binders, which are able to attach to specific DNA sequences (Beisel et al., 2002; Papoulas et al., 1998).

The mechanism of action of TrxG is similar to that of PcGs and TrxG are able to recognize Trithorax response elementes (TREs) as well as PREs, meaning that they can be recruited to the same loci as the PcGs (Chinwalla et al., 1995). However, TrxG action is antagonistic to that of PcGs, helping to maintain expression of targeted genes (Petruk et al., 2008). This antagonism has been demonstrated with the help of

different PcG and TrxG mutants, showing how when PcG genes are mutated, there is an enhanced phenotype of the TrxG mutants (Gilden et al., 2000; LaJeunesse & Shearn, 1996).

1.4.1.5 Bivalent chromatin

PcG and TrxG proteins play a fundamental role in maintenance of the pluripotent state of ES cells (O'Carroll et al., 2001). The study of many of the pluripotency genes expressed in ES cells, at the genome-wide level, has shown the presence of markers for active transcription, such as H3K4me3. At the same time, promoters of some of the genes were enriched in H3K27me3 (repression) (Bernstein et al., 2006; Chambeyron et al., 2005). Deeper analysis using ChIP confirmed that these active and inactive histone marks were present in the same physical space, a phenomenon termed “bivalent (poised) chromatin” (Bernstein et al., 2006). This situation is found in highly conserved ES genes and transcription factors of the Sox, Pax, Po, Fox and Irx families, and it seems to be the hallmark of genes that are ‘poised’ to be quickly switched on/off when required for cell development such as differentiation (Figure 1.5) (Spivakov & Fisher, 2007). Moreover, mammalian GC are enriched in bivalent chromatin at promoters of different genes that regulate the somatic fetal development. This poised state in the fetal germ line is maintained from sexual differentiation and during the postmeoitic stages and they have been proposed to help in the maintenance of GC identity as well as preparing the gametes for the totipotency stage following fertilization (Lesch & Page, 2014; Lesch et al., 2013).

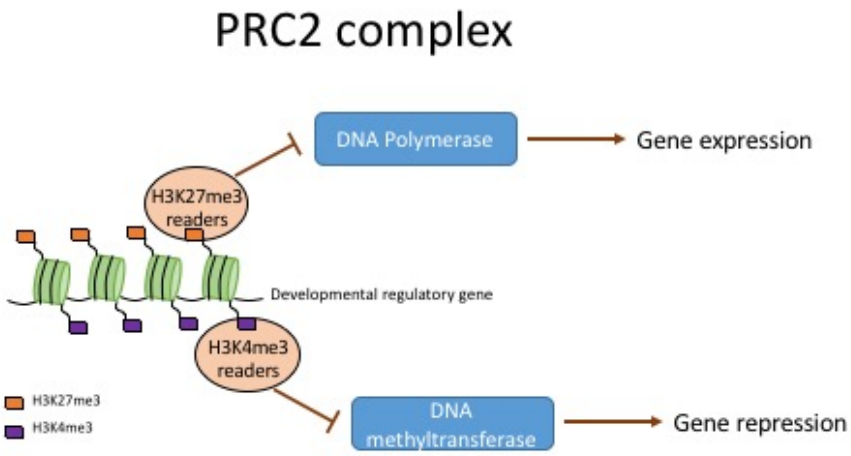


Figure 1.5: The bivalent chromatin state. H3k27me3 and H3k4me3 produce opposing effects on the expression of their target genes. Both markers can be present in the same genes, but H3K27me3 involves the repression of the genes, while H3K4me3 promotes their expression.

1.4.2 DNA methylation

The methylation of DNA is probably the most studied epigenetic modification. In this case, a methyl group is added directly to a DNA base. In eukaryotes, the methylation can only be added to a cytosine, although methylated adenosine can be found in prokaryotes (Jin et al., 2011; Low et al., 2001). The role of DNA methylation is to regulate gene expression by affecting chromatin structure and interfering with the binding of transcription factors (Siegfried & Simon, 2010). This role was first demonstrated in *Xenopus Laevis* oocytes by the injection of in vitro methylated DNA and showing repression of gene expression (Stein et al., 1982; Vardimon et al., 1982).

DNA methylation is able to target many different families of genes and it plays a key role in normal cell development. It is especially relevant during embryo development, and mice with inactivating mutations in the DNA methyltransferase genes responsible for de novo DNA methylation, do not survive fetal life (Li et al., 1992; Okano et al., 1999).

1.4.2.1 CpG islands

DNA methylation is found all across the DNA of the genome. However, the majority, around 75%, is found at Cytosine-phosphate-Guanine (CpG) sites. CpGs are usually associated in clusters known as CpG islands (CGIs), discovered more than 30 years ago by Adrian Bird (Bird, 1986). CpG dinucleotides are hence unevenly distributed, as they are over-represented in CGIs but are less evident in the rest of the genome (Majewski & Ott, 2002). The majority of CpG dinucleotides are methylated, although this does not occur in GCIs, which show low levels of methylation (Strichman-Almashanu et al., 2002). It has been shown that CpGs are more highly conserved among different species if they are located within CGIs. This can be explained because methylated CpGs can derive easier into uracil, which is less stable (and less maintained) than the thymine produced by the methylation of unmethylated cytosine. This means that methylation regions have higher chances of losing CpG due to mutations (Duncan & Miller, 1980; Tanay et al., 2007; Yang et al., 2014). This different mutation rate has also been found within CGIs, comparing methylated with non methylated CpGs (Bock et al., 2006).

Many genes regulated by DNA methylation show CGIs associated with their promoters (Babenko et al., 2017). Methylation of these CGIs is usually linked with reduced transcription, although, when unmethylated, they are not always associated with active gene transcription (Schübeler, 2015). Moreover, a recent paper disclosed a mechanism with the presence of alternative distal promoters, formed by CGI, and allowing the transcription of genes with silenced proximal promoters (Sarda et al., 2017).

1.4.2.2 Imprinted genes

Each cell in our body has two copies of each gene, one from each parent. However, both copies of each gene do not necessarily need to be expressed. The mechanism by which only one of the copies of specific genes are expressed, depending on their

parental origin, is known as imprinting. This mechanism was first described in 1984 by two simultaneous publications showing how pronuclear exchange between fertilized zygotes resulted in abnormal embryos (with two paternal or maternal genomes) (Barton et al., 1984; McGrath & Solter, 1984). Since then, over one thousand imprinted genes have been described (Kelsey and Bartolomei., 2012). The silencing of one of the copies of an imprinted gene is coordinated by short sequences of DNA (imprinting control regions), which exhibit different DNA methylation depending on the parental origin of that gene (Bajrami, 2016). Moreover, many imprinted genes are localized together in the genome, forming clusters (Ferguson-Smith, 2011). The correct imprinting pattern in these clusters is regulated by the imprinting control region (ICR), a genetic element controlled by different epigenetic regulatory factors. The ICR is responsible of the asymmetry found in imprinted genes within eggs and sperm, but also for keeping that asymmetry after fertilization and allowing a different transcription between alleles in the same nucleus (Hanna & Kelsey, 2014; Proudhon et al., 2012).

Another imprinting system present in mammals is X chromosome inactivation. In most mammals, females carry two copies of the X chromosome and males carry the Y chromosome, which contains the *SRY* gene along with other genes important for spermatogenesis and sex determination processes. However, this asymmetry causes a dosage imbalance, especially important for the genes of the X chromosome. Genes from the X chromosome need to be regulated to ensure a balance between the sexes, but also between these genes and autosomal ones. Nature has solved this problem by the inactivation of a whole X chromosome in cells from female individuals, first proposed in 1961 (Lyon, 1961). The inactivation starts after fertilization by the accumulation of X-inactive specific transcript *(XIST)*, a non-translated RNA that coats the X chromosome. This process is followed by other epigenetic changes which contribute to whole chromosome silencing (Penny et al., 1996). *XIST* is present in many mammals, however others such as marsupials rely on an Xist-like gene able to

produce a similar effect (Grant et al., 2012). The inactivated X chromosome is more dense than other regions of the chromatin making it visible in somatic cells, which is called the Barr body (Lyon, 1961).

1.4.2.3 DNA methyltransferases (DNMTs)

DNA methylation is a dynamic process in which methyl groups are added to cytosines by the activity of enzymes known as DNMTs. Different classes of DNMTs have been identified: DNMT1 is in charge of maintaining established DNA methylation whereas DNMT3 enzymes induce *de novo* methylation of unmethylated CpGs (Lei et al., 1996; Okano et al., 1999). There is a third DNMT described, DNMT2, although it does not have a role in DNA methylation, but in tRNA methylation (Dong et al., 2001).

1.4.2.3.1 DNMT1

When a cell synthetizes a new strand of DNA for cell division, this strand needs to be methylated to follow the same pattern as the original one. DNMT1 is in charge of this maintenance of DNA methylation during replication by copying the information from one DNA strand to the other. This methyltransferase was the first of the DNMTs to be discovered (Bestor et al., 1988) and started a whole new field in understanding the process of DNA methylation. DNMT1 is associated with the replication foci targeting sequences (RFTS), which are able to attract the enzyme to replication sites (Leonhardt et al., 1992). DNMT1 has a higher affinity for hemi-methylated DNA (one strand is methylated only). This affinity allows the enzyme to methylate hemi-methylated DNA over 20 times faster than fully methylated or unmethylated DNA. Moreover, DNMT1 can only methylate in one orientation with respect to the DNA, allowing it to methylate only one strand of DNA (Hermann et al., 2004).

DNMT1 activity is assisted by other enzymes, such as NP95 (also known as RING finger1 or Uhrf1) and Proliferating cell nuclear antigen (PCNA) (Bostick et al., 2007;

Sharif et al., 2007). NP95 localizes and binds hemi-methylated DNA thanks to its SET- and RING associated domain before recruiting DNMT1 (Arita et al., 2008; Sharif & Koseki, 2011). The role of NP95 also entails histone modifications. Apart from being able to recognize H3K9 trimethylation (H3K9me3) (Arita et al., 2012), it has a RING finger domain able to ubiquitinate histone H3 at H23 or 18, to which DNMT1 will preferentially bind (Nishiyama et al., 2013; Qin et al., 2015). PCNA also promotes localization of DNMT1 to the RFTS together with NP95, although its presence is not necessary for normal DNMT1 functioning (Spada et al., 2007). Modifications in the DNMT1/PCNA/NP95 localization system is related to decreased DNA methylation and is associated with tumourgenesis (Hervouet et al., 2010; Pacaud et al., 2014).

1.4.2.3.2 DNMT3

DNMT3 enzymes are responsible for the de *novo* methylation of unmethylated CpGs. Three different DNMT3s have been described: DNMT3A, 3B and 3L (Holz-Schietinger & Reich, 2010; Okano et al., 1999). Unlike DNMT3A and 3B, DNMT3L lacks the catalytic domain to transfer the methylation to the cytosine (Holz-Schietinger & Reich, 2010). Studies have shown that DNMT3A and 3B target different loci across the genome, although there is some level of compensation of activity (Kato et al., 2007). DNMT3A and 3B are essential for normal development and knockouts are highly detrimental. Knockout mice for Dnmt3a present with poor growth and normally die in the first 4 weeks after birth, while Dnmt3b knockout mice die earlier, at e9.5 during fetal life (Okano et al., 1999). Dnmt3l knockouts are viable, but show a reduced rate of early methylation in the zygote which specifically affects some regions in the genome (Guenatri et al., 2013). Dnmt3L is also essential for proper reproductive function and, when disrupted, it is associated with a phenotype of azoospermia, but also with failures during imprinting of the imprinting loci (Bourc'his & Bestor, 2004; Bourc'his et al., 2001).

1.4.2.4 TET proteins

The Ten-eleven translocase1 (TET1) gene was described for the first time in acute myeloid leukaemia, as it was common to find a translocation in t(10;11)(q22;23), in these patients, where TET1 is located (Lorsbach et al., 2003). Later on, two other members of the same family were described, TET2 and TET3.

Different publications have demonstrated that TET1 is a key factor in the process of DNA demethylation, oxidizing the 5-methylcytosine (5mc) to 5-hydroxymethylcytosine (5hmc) (Gong & Zhu, 2011; Zhu, 2009) (Figure 1.6). Tet1 can be found in transcription start sites, but also at promoters of PcG target genes (Williams et al., 2011). The mechanism of Tet1 also involves the conversion of 5hmc into a cytosine by oxidations steps of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Both, 5fc and 5caC can then be replaced by a cytosine by the base excision repair (BER) pathway, with the action of a thymine DNA glycosylase (TDG) (He et al., 2011; Maiti & Drohat, 2011). Tet1 knockdown in mice results in an increase in global methylation and a reduction in 5hmC (Ito et al., 2010). Using a gene trap derived mutant of Tet1 (showing 5% of Tet1 expression), researchers showed reduced animal viability and reduced litter size. Within the survivors, males showed a normal testis, although females had ovaries that were reduced in weight (Yamaguchi et al., 2012), usually a sign of reduced reproductive function/fertility.

TET2 usually co-localizes with TET1 and their roles can be redundant in some cases (Huang et al., 2014). TET3 has only been described in the zygote and the oocyte (Wossidlo et al., 2011). Depletion of TET1 in a carcinoma cell line showed reduced 5hmc, while TET2 and TET3 depletion decreased 5hmc in several TET1 targets (Kang et al., 2015). Other experiments have shown that TET2 and TET3 are actually able to catalyze the same reactions as TET1 (Ito et al., 2010; Wossidlo et al., 2011). TET2 mutants are viable, but they showed reduced 5hmc and problems with

haematopoiesis. Combined knockout of TET1 and TET2 results in even greater loss of 5hmc, fitting with the idea that both can normally compensate in part for each other. The double knockout animals are viable, but suffer high perinatal mortality and surviving adults show a decrease in gonadal size (Dawlaty et al., 2013). TET3 has been shown to be related to demethylation of paternal imprints after fertilization. Its knockdown produces embryonic lethality and no expression of pluripotency genes from the paternal genome (Gu et al., 2011; Wossidlo et al., 2011) .

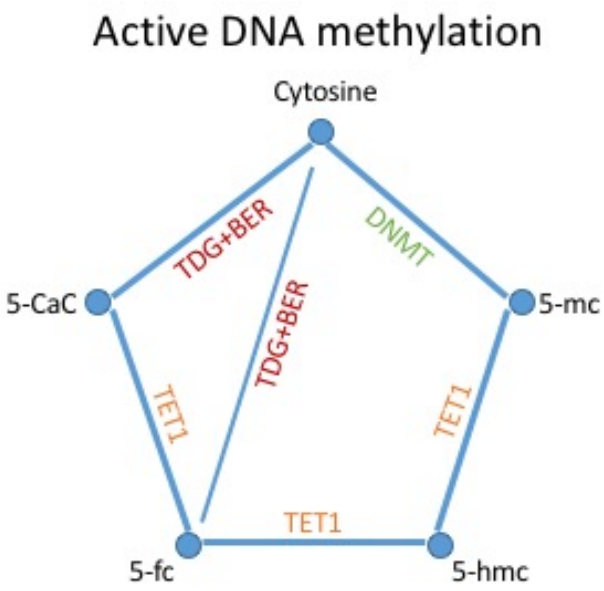


Figure 1.6: Active DNA demethylation by TET1. The process of removing a methyl group from a cytosine is carried in different steps by TET1. First, 5mC is converted into 5hmc, which is transformed into 5-fc and consequently into 5CaC (not needed), all steps catalysed by TET1. A final step is performed by the base excision repair machinery (BER) in order to obtain the non methylated cytosine.

1.4.3 Cross-talk between DNA methylation and H3K27me3

Among all the mechanisms of gene silencing, DNA methylation and H3K27me3 are probably the most common ones. They are both involved in the establishment and maintenance of gene repression and different experiments have shown evidence of the presence of a cross-talk between PRC2/H3K27me3 and DNA methylation. For

example, EZH2 is able to positively regulate DNA methylation, as it was able to interact with DNMTs involved in DNA methylation of different promoters targeted by EZH2 (Viré et al., 2005). Other studies have suggested that this relationship might be expressed in form of antagonism. As a consequence of *Dnmt3a* deficiency in mice neural stem cells, there was an increase in H3K27me (Wu et al., 2010). Deeper analysis using ES cells from triple DNA methyltransferases (DNMT1, DNMT3A and 3B) mice knockouts, where DNA methylation is reduced, indicated that hundreds of thousands of genes normally methylated showed the presence of H3K27me3 instead. However, in the opposite scenario, where PRC2 is lost, only a small percentage of genes normally regulated by H3K27me3 showed DNA methylation (Hagarman et al., 2013). Interestingly, this relationship between DNA methylation and H3K27m3 might be disrupted in cancer cells. Genes containing CpG islands, which are PcG targets, tend to lose H3K27me3 and acquire DNA methylation silencing (Gal-Yam et al., 2008; Schlesinger et al., 2007).

1.4.4 Epigenetics of germ cells

Cell development is highly related by epigenetic processes. From the zygote stage, cells undergo dramatic modifications to their epigenome. These epigenetic modifications produce unique profiles in each cell type and will define the future cells and tissues of the body by differential, cell-specific gene expression. Hence, during differentiation of cells from a pluripotent state into a differentiated state (eg. somatic cells), specific epigenetic modifications result in a more restricted gene expression profile until they are “locked” into their differentiated state (Reik, 2007). However, this epigenetic remodelling remains highly active in GC, from their initial differentiation until their final transformation into gametes (Hajkova et al., 2008). Moreover, GCs show a unique epigenetic machinery, involving particular proteins to undergo specific epigenetic remodelling (Hajkova et al., 2008; Santos & Dean, 2004). The majority of the studies on the epigenetics of GC are based on studies in *Drosophila* and in mouse models.

1.4.4.1 DNA methylation in germ cells

Since the early beginning of PGC differentiation, GCs already show epigenetic differences compared with the somatic cells surrounding them. Studies in *Drosophila* showed that PGCs exhibit low levels of the activational H3K4me3 mark and high levels of H3K9me, a marker of repression. These histone modification patterns suggest that these cells are probably repressing the somatic cell gene expression program (Schaner et al., 2003). In mice, in which PGC fate is established at e7.5, repressive markers, such as genome-wide DNA methylation, levels of H3K9 dimethylation (H3K9me2) and H3K27me3, are similar to somatic cells. However, DNA methylation levels start to decrease after e8.0. The global decrease in DNA methylation in PGCs does not affect all DNA regions and there are mechanisms to protect specific genes from DNA demethylation. Mvh, Dazl and Sycp3, important PGC markers that are only expressed after migration to the genital ridge, and DNA methylation analysis in mice showed methylated flanking regions in these genes by e10.5. However, these genes undergo demethylation and are expressed by e13.5 (Maatouk et al., 2006). Further analysis, using DNMT1 mutant embryos, showed expression of these genes by e9.5, suggesting an important role of DNMT1 in maintenance of gene repression in earlier stages of development (Maatouk et al., 2006). Another factor playing a role in DNA methylation is STELLA, which is expressed in PGCs at e7.0-e7.5. STELLA has been shown to protect and maintain the methylation state of PGCs and the methylation of the maternal genome in zygotes by inhibiting the conversion of 5mC into 5hmc, which preserves maternal chromosome integrity (Nakamura et al., 2007; 2012; Nakatani et al., 2015).

After arriving to the genital ridge (e9.5 in mice), PGCs undergo a second wave of extensive epigenetic reprogramming. This process includes the erasure of parental imprinting and reactivation of the inactive X chromosome (females only). The demethylation of the imprinted paternal loci takes place quickly, between e10.5 and e12.5, which points to an active process (Hajkova et al., 2002; Lee et al., 2002). The

activation of the X chromosome takes place in a longer time window, starting during PGC migration and finishing in the post-migratory phase (de Napoles et al., 2007).

Once sex determination has been initiated, PGCs start to re-establish DNA imprints. At this stage, male and female PGCs show morphological and functional differences and their epigenetic patterns diverge as well. These distinctive patterns are probably a reflection of the different developmental paths that GCs will undergo: female GCs will be arrested in meiotic prophase while male GCs are arrested in G1 of mitosis at this time. Male GCs progressively re-establish methylation between e14.5 and birth (Reik, 2001), including paternal imprinted loci, thanks to de novo methylation by DNMT3s. Conditional knockout of DNMT3a and b in GC, reveals that DNMT3a is able to re-methylate all of the paternally imprinted genes, but DNMT3b only plays a role in the methylation of Rasgrf1 (Kaneda et al., 2004). Female GCs exhibit a temporal delay in DNA re-methylation imprinting, as It does not start until birth, during oocyte growth, and is only completed when the oocyte is fully grown (Hiura et al., 2006); conditional knockouts have shown essential roles for DNMT3A and DNMT3B, but not for DNMT3L, in this process (Kaneda et al., 2004).

1.4.4.2 Histone modifications in germ cells.

Histone modifications in PGC have been investigated mainly in mice, and are mainly represented by H3K9me2 and H3K27me3, but also by H3K4me3. H3K9me2 levels start to decrease after e7.5, just before the decrease in DNA methylation. By e8.75, the presence of H3K9me2 is clearly lower in PGC than in the surrounding somatic cells (Seki et al., 2005; 2007). On the other hand, H3K27me3 undergoes the opposite change and is up-regulated by e8.25 and significantly increased by e9.5 (Seki et al., 2007). The presence of H3K4me3 and H3K27me3 in PGC suggests the possibility of bivalent chromatin regulation of pluripotency genes (Bernstein et al., 2006). Experiments using ChIP, showed that domains containing H3K4me3 and H3k27me3 are highly enriched in mice at developmental regulatory genes in a similar way to

ESCs (Sachs et al., 2013). Moreover, the same publication was able to show that during sexual differentiation, at e13.5, these developmental genes stay silent and bivalent (Sachs et al., 2013). New models have helped to understand the epigenetics of PGC specification, developing PGC-like cells (PGCLCs) from ESC-derived epiblast-like cells (EpiLCs). This model showed that EpiLCs contain low H3K27me3 levels in bivalent gene promoters, while PGCLCs lose H3K4me3 in these promoters with an increase in H3K27me3. (Vierstra et al., 2014). Furthermore, PGCLCs have low levels of H3K9me3 (Liu et al., 2014; Matsui & Mochizuki, 2014). The processes of histone modifications and DNA demethylation in PGCs seems to share common mechanisms. H3K9me2 can bind STELLA directly and inhibition of H3K9me2 produces a decrease in DNA methylation levels by failure of STELLA recruitment (Nakamura et al., 2012).

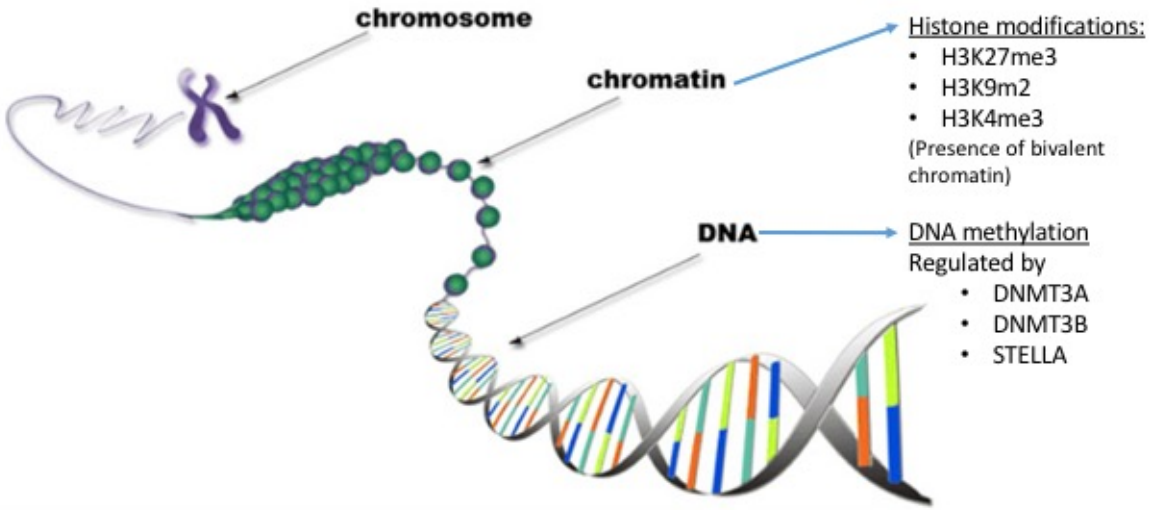


Figure 1.7: Gene silencing in Germ cells. The regulation the epigenetic marks are key during GC development. GCs present a cell specific epigenetic machinery, distinct from other cell types. Within histone modifications, there is a high presence of bivalent chromatin, including genes regulated by H3K27me3 and H3K4me3. DNA methylation regulation is important to maintain their pluripotent state.

1.5 Common analgesics

1.5.1 Paracetamol

Paracetamol, is probably the most commonly used analgesic around the world, and its consumption has increased progressively during the last decades (Kristensen et al., 2016). Introduced into the pharmaceutical market in 1955, it is an over the counter drug (no prescription needed) and its consumption has been generalised within our society. It is recommended as a first line treatment for fever and pain associated with many different conditions, including cancer. It is also the most common choice for children, and patients unable to use NSAIDs, such as people with bronchial asthma, haemophilia, peptic ulcer disease or sensitivity to salicylates (Jóźwiak-Bebenista & Nowak, 2014).

However, despite the wide use of paracetamol in medical practice, its mechanism of action remains mainly unknown (Smith, 2009). It shares some characteristics with NSAIDS, such as its anti-pyretic properties, although it does not show anti-inflammatory activity like NSAIDs. The other point in common with NSAIDS, and which will be highly discussed in this thesis, is the ability of paracetamol to supress prostaglandin (PG) production/action. How paracetamol affects the PG pathway is still uncertain. Some studies suggest an effect on cyclooxygenase (COX) 1 and COX2 enzymes, with a consequence reduction in PG production. There are two main forms of COX enzymes: COX1 and COX2. The former can be found in most cells, while the latter is predominantly expressed during early stages of cell differentiation or replication. COX2 has been reported to also be expressed in physiological processes such as inflammation, bone absorption and angiogenesis, but also in pathological ones, such as female genital tract disorders, kidney diseases or brain disorders(Katori & Majima, 2000) and is over-expressed in many types of cancer (Harris et al., 2002).

However, not all studies have shown an effect of paracetamol on COX enzymes and some others have suggested that it might have a role in blocking PGE2 or its receptors (Anderson, 2008; Hutson et al., 2012; Pickering et al., 2006; Sandrini et al., 2007).

Another possibility to explain some of the pharmacological effects of paracetamol, independently of PGs, is an alteration in the serotoninergic pathways, which play a role in inhibition of pain perception. One study on serotonin receptors using rat models showed that paracetamol has an effect on the 5-HT subtype receptor (Alloui et al., 2002). Another study, this time in human, showed that paracetamol was able to block the pain produced by electrical stimulation of the median nerve (Pickering et al., 2006; Pickering et al., 2008).

1.5.2 NSAIDS

NSAID drugs are a group of drugs that act as analgesics (pain-relief), anti-pyretics (reduce fever) and anti-inflammatories. The most common NSAIDs are ibuprofen and aspirin (acetylsalicylic acid), which apart from paracetamol, are the most common drugs used worldwide. Similar to paracetamol, the consumption of ibuprofen and aspirin is increasing in our society (Kristensen et al., 2016). Ibuprofen was introduced into UK in 1969 and worldwide during the 1970s. It is often prescribed as a first line NSAID instead of other NSAIDS, such as aspirin, indomethacin or phenylbutazone (Rainsford, 2003) and is also available over the counter without prescription.

NSAIDS are used to control fever, acute pain and acute inflammatory reactions. These therapeutic effects are mediated via inhibition of COX1 and COX2 (Burian & Geisslinger, 2005; Hinz & Brune, 2006; Rainsford, 2003). Effects on pain relief are attributed to their anti-inflammatory effects on the peripheral and central nervous system (Martinez et al., 2002). The antipyretic role of NSAIDs are mainly caused by

their effects on PGE_2 synthesis, which alters the neuronal process able to control thermoregulation in the hypothalamus (Aronoff & Neilson, 2001).

1.5.3 Prostaglandins

As outlined above, one potential mechanistic pathway that might link analgesics and epigenetic alterations is the PG pathway. PGs are lipid compounds, whose physiological activity is similar to hormones, but acting in a more auto/paracrine way, being able to target themselves and neighbour cells. They were first discovered in seminal fluid in 1935 and received that name because they were thought to be a prostatic-derived compound (Euler, 1935). Later on, many other PGs were discovered and categorized in different tissues. Further research has found PGs in the majority of human tissues. Unlike hormones, PGs are produced in different places throughout the body and not in a specific site (Kalinski, 2012). PGs have been related to a wide variety of mechanisms, such as cell growth (Yun et al., 2009), calcium movement (Seifi et al., 2015), contraction and relaxation of vascular smooth muscle cells (Markiewicz et al., 2016), kidney filtration (Pugliese & Ciabattoni, 1984), platelet disaggregation (Kikura et al., 2000), sensitivity of neurons (Latremoliere & Woolf, 2009), body temperature (Veltmeijer et al., 2016) and parturition (Rugarn et al., 2016) among many others.

The synthesis of PGs involves COX1 and 2 enzymes. These cyclooxygenases convert arachidonic acid to PGG, which is later reduced to PGH by the same enzymes. PGH is then converted to an active PG by different synthases and reactions of dehydration or ketoreductases (Frungieri et al., 2004). So far, four principal types of PGs have been described: PGE_2, PGI_2, PGD_2 and $PGF_{2\alpha}$. These can activate different receptors, which are G-protein coupled (Table 1.5). Different second messengers have been described for the different receptors, with an increase in cyclic adenosine monophosphate (cAMP) being the most common (Ricciotti & FitzGerald, 2011).

Table 1.5: Signal Transduction of prostanoid Receptors

Class	Subtype	G-protein coupled	Second messenger
PGE_2	EP1	G_q	$\uparrow IP_3$
	EP2	G_s	$\uparrow cAMP$
	EP3	G_i, G_{12}, G_{Rho}	$\downarrow cAMP$, $\uparrow Ca^{2+}$
	EP4	G_s	$\uparrow cAMP$
PGD_2	DP	G_s	$\uparrow cAMP$
	CRTH2	G_i	$\downarrow cAMP$, $\uparrow Ca^{2+}$
$PGF_{2\alpha}$	FP_A, FP_B	G_q, G_{Rho}	$\uparrow IP_3$
PGI_2	IP-IP	G_s, G_q, G_i	$\uparrow\downarrow cAMP$, $\uparrow IP_3$
	IP-TP_α	G_s	$\uparrow cAMP$
TxA_2	TP_α, TP_β	G_q, G_s (α), G_i (β)	$\uparrow IP_3$, $\uparrow\downarrow cAMP$
		G_h, $G_{12/13}$	$\uparrow Ca^{2+}$

The role of PGs in fertility was found thanks to the study of *Cox* knockout mice. Female *Cox2* knockout mice are infertile, while *Cox1* knockout females produce normal weight litters. However, these models showed no effect on male fertility (Langenbach et al., 1999). Studies on the different PGs, in mice and human, have described the presence of different receptors in the different testicular cell types: EP1,DP,FP and TP in the Leydig cells (Frungieri et al., 2006; Kowalewski et al., 2009; Pandey et al., 2009; Schell et al., 2007; Walch et al., 2003), EP1,EP2,EP3,EP4,DP,IP and FP in Sertoli cells (Ishikawa & Morris, 2006; Matzkin et al., 2012; Winnall et al., 2007) and PARP in the seminiferous tubule wall (Frungieri et al., 2002). Recent studies on fetal gonads have shown the presence of EP2 receptors and COX2 in both somatic and GCs in the rat (Dean et al., 2016). The expression of PG receptors in the ovary has also been studied. All PGE_2 receptors, as well as FP are expressed in follicles or the corpus luteum and different roles for each of them have been hypothesized (Graves et al., 1995; Kim et al., 2014). COX1, COX2, EP2 and EP4 are also expressed in the fetal human ovary, with COX1 being restricted to somatic cells and EP2, EP4 and COX2 to germ cells (Bayne et al., 2009). Ep2 and Ep4 are expressed in germ cells

in the rat ovary (Dean et al., 2016). The actual role of PGs in fetal gonad development is still largely unknown. However, the presence of different PG receptors in bot, somatic and germ cells, suggest that they might play an important role in different stages of ovary and testis development. For example, PGE_2 has been shown to be important in Sox9 regulation during fetal testis development. When *Sox9* is expressed in the male differentiating gonad, it activates the expression of prostaglandin D synthetase (Pgds), which is also able to enforce *Sox9* expression (Wilhelm et al., 2007a).

Paracetamol, indomethacin and ibuprofen all target PG pathways, and somatic and germ cells in the fetal gonads of both sexes in rats and humans are sources and targets of PGs as they express the COX2 enzyme and PGE2 receptors (Bayne et al., 2009; Dean et al., 2016). Numerous studies have shown that PGE_2 exerts a range of effects on normal and cancerous cells, in vivo and in vitro. These result in altered cell proliferation (Yun et al., 2009), stem cell pluripotency (Yun et al., 2012) or alterations in epigenetic markers, such as DNA and histone methylation, but also changes in the expression of key epigenetic regulatory factors, such as DNMT3a and b or EZH2 (Arosh et al., 2015; Venza et al., 2012; Xia et al., 2012). These are discussed in more detail in section 1.5.3.3 below.

1.5.3.1 Prostaglandins and cell proliferation

Among the different roles of PGs, one that is common within different cell types is the promotion of cell proliferation. Increased cell proliferation has been reported after the addition of PGE_2 in a wide variety of cell types, such as fibroblasts, myocytes, T-cells, hepatocytes or skeletal muscle myoblasts (Kimura et al., 2001; Krause et al., 2007; Mendez & LaPointe, 2005; Mo et al., 2015; Sanchez & Moreno, 2002). Studies on the mechanism used by PGs to increase proliferation have revealed a variety of pathways involved. A lot of attention has been focused on the capacity of cAMP, production of which is stimulated by PGs, to affect cell proliferation via interaction

with the mitogen activated protein kinase (MAPK) cascade, a well studied mechanism (Stork & Schmitt, 2002). However, further studies have shown that this interpretation is not this simple, and different cell types show different mechanisms involving the different PGE_2 receptors. Hence, the downstream effects are even more varied. For example, experiments on cultured myocytes showed how PGE_2 was able to activate the epidermal growth factor receptor (EGFR) via EP4. This resulted in activation of MAPK which modified cell proliferation (Mendez & LaPointe, 2005). Similar experiments using hepatocytes revealed a different mechanism, as PGE2 induced cell proliferation was mediated by EP1 activation, which increased the secretion of TGF-α, affecting the tyrosine kinase /MAPK pathway (Kimura et al., 2001). Moreover, PGE_2 is able to modify more than one of these mechanisms in the same cell type. Studies on fibroblasts using specific antagonists for EP1 or EP4 receptors revealed a similar phenotype of cell reduction by both treatments, but via different mechanisms. The EP1 inhibitor was able to arrest cells in G0/G1 phase, while the EP4 inhibitor arrested the cells in the synthesis phase (Sanchez & Moreno, 2002). Other studies have performed experiments targeting different EPs and their role in proliferation. Studies on skeletal muscle myoblasts using PGE_2, and combinations of agonists/antagonists for the different EPs, was able to show that proliferation was only modified by targeting EP4 (Mo et al., 2015).

There has also been a big focus on the role of PGs on the proliferation of different cancer cell types, such as colon cancer, lung cancer, gastric cancer, laryngeal papilloma, breast cancer or endometrial cancer amongst others (Bhooshan et al., 2016; Ke et al., 2016; Nandi et al., 2017; Ren et al., 2016). Many of these cancers exhibit increased COX-2 expression and PGE_2 production compared with their respective healthy tissue (Ke et al., 2016; Ren et al., 2016; Zelenay & Sousa, 2016). Similarly to non-tumoural cell types, the mechanism of PGE_2 action on cancer cells seems to vary according to the model used. One study, using human laryngeal papilloma, correlated the PGE_2-mediated increased proliferation with activation of

MAPK. Moreover, blocking of the COX2/MAPK pathway with isoflurane resulted in reduced proliferation and increased apoptosis (Ren et al., 2016). Different studies have linked PGE_2-mediated changes in proliferation with the EP4 receptor. In vitro studies using COX-2-expressing rodent breast cancer cell lines, showed how proliferation and migration was mediated by COX2, PGE_2 and the EP4 receptor. COX2 inhibitors and EP4 antagonists were able to significantly reduce proliferation and migration with a consequent reduction in tube formation, which is characteristic of this cancer cell line. These effects produced by a COX2 inhibitor and EP4 antagonists were reversed by the addition of PGE2 and EP4 agonists. Similar results were found when these cell lines were treated with EP4 receptor siRNA (Nandi et al., 2017). Another study found a relationship between the use of EP4 agonists and overall survival or recurrence of lung cancer (Bhooshan et al., 2016). One study, using endometrial cancer cells, also showed increased cancer cell proliferation as a result of PGE_2 exposure. Further analysis found that enhanced proliferation was mediated by the EP4 receptor and small ubiquitin-related modifier 1 (SUMO-1), and was related to activation of the Wnt/β-catenin signalling pathway (Ke et al., 2016).

Proliferation of different human stem cell types have also been shown to be altered by PGs. Diverse publications have studied how PGs can promote proliferation in undifferentiated and differentiating neuroectodermal stem cells, adult stem cells from cord blood or adipose tissue, tendon stem cells, as well as in mice embryonal stem cells (mESCs) (Lee et al., 2016; Wong et al., 2014; Wong et al., 2016; Yun et al., 2009; Zhang & Wang, 2014). Increased proliferation in these cell types has been linked to modifications in the wnt/βcatenin pathways as well as to other downstream alterations, such as PKA, a common target of cAMP (Wong et al., 2016). The increased proliferation in stem cells is relevant in the context of the gonads because ESCs and GC exhibit similarities in many of their characteristics. However, probably the most similar stem cell type are the mESCs. PGE_2 exposure of these cells was able to significantly increase thymidine incorporation and cell number. Deeper analysis

showed how PGE2 was also able to modify a diverse range of cyclins, thus providing a mechanism whereby the cell cycle and hence, cell proliferation might be effected (Yun et al., 2009).

1.5.3.2 Prostaglandins and pluripotency

The literature also shows that PGs, apart from affecting proliferation of stem cells, can also alter their pluripotency state. Different studies have shown that exposure to PGE_2 could modify the expression of differentiation markers and modify the pluripotency state. Exposure to PGE_2 in neuroectodermal stem cells resulted in modifications of the expression of Oct4 (stem cell marker) and Mapt (neuronal differentiation marker) (Wong et al., 2016). Similar experiments performed in tendon stem cells, rat retinal Müller glial stem cells or chondrocyte stem cells, showed that PGE_2 was able to modify expression levels of tenocyte related genes (Collagen I and Tenascin C), retinal stem cell markers (Pax6) or chondrogenic differentiation markers (Colagen type 2; Col2a1 or Aggrecan; Acan) respectively (Caron et al., 2016; J. Zhang & Wang, 2014). PG exposure of mESCs also showed an effect on their pluripotency state. When exposed to PGE2, the expression of the well-known mESCs differentiation markers Sox2, Nanog and Oct4, was reduced (Yun et al., 2009). In a more relevant model for this thesis, one paper using human testicular explant cultures has shown how ibuprofen, which was able to reduce PGE_2 levels in the samples, was also able to modify the expression of certain GC markers, such as OCT4 and Lin28 (Ben Maamar et al., 2017).

1.5.3.3 Prostaglandins and epigenetics

PGs have also been associated with alterations in epigenetic markers in a diverse range of studies. Whether these alterations are a cause or a consequence of the other phenotypes reported, is not well studied. Modifications of DNA methylation are probably the most studied phenotype of PGs within the epigenome, and have been linked with modifications to expression of the different DNA methylation

regulatory factors, such as DNMTs. PGE_2 has the ability to silence, via DNA methylation, certain tumor suppressor and DNA repair genes which promoted intestinal tumor growth, suggesting a relationship between this and the mentioned role of PGs in cell proliferation. Further analysis using celecoxib (NSAID, COX2 inhibitor) showed how the PG-induced changes in DNA methylation were driven by modified expressions of DNMT1 and DNMT3B (Xia et al., 2012). Another study using a mouse model found that exposure to UV radiation resulted in increased levels of COX2, PGE_2 and EP receptors in the skin. UV radiation was also able to induce DNA hypermethylation as a result of increased levels of the different Dnmts. All of these responses were down-regulated when the skin was treated with indomethacin or EP2 antagonists, demonstrating that the modifications in DNA methylation were driven by the PGE_2 pathway (Prasad & Katiyar, 2013). Other studies have tried to study the DNA methylation modifications in more detail, including mechanistic studies. One study, using fetal and adult fibroblasts, was able to identify multiple genes that were hypermethylated in a specific manner as a response to PGE_2 exposure, independently from global methylation. Apart from finding an increase in global DNA methylation, this study used array techniques in order to identify specific genes that were methylated as a consequence of exposure to PGE_2, and were able to identify 18 such genes, including some related to DNA and histone methylation (RUNX3, SFRP5 and JMJD2A), genes related to cell proliferation (cyclin CCND1 and TBX2), as well as tumour suppressor genes (LATS2 and LLGL1). Deeper analysis of the mechanistic modifications found that these changes in methylation were a result of modifications in DNMT3a activity and revealed other modifications, such as increased expression of specificity proteins 1 (SP1) and 3 (SP3). SP1 and SP3 are transcription factors known to control the expression of a wide variety of genes, including DNMT3a (Huang et al., 2012).

Modifications of the epigenome as a result of PG exposure is not restricted to DNA methylation, but also applies to histone modifications. At least one publication using

endometriotic cells has shown that exposure to EP inhibitors resulted in variations in the histone modifications markers, as well as changes in the expression of key epigenetic regulatory factors. This publication showed how using a combination of EP2 and EP4 antagonists, there was a decrease in expression of DNMT3a and DNMT3b, suggesting a decrease in DNA methylation. Moreover, EP antagonists were able to modify a wide variety of histone modifications, including H3K9ac, H3K9me3, H3K9me3:ac, H3K27me3 and H3K27me3:ac. Furthermore, this publication showed effects of EP antagonists on the expression of a wide variety of epigenetic regulatory factors, including EZH2, SUV39H1, JMJD2A and HDAC1 (Arosh et al., 2015).

1.6 Summary

Despite the increasing number of studies relating fetal changes to the intake of over the counter analgesics, such as paracetamol, ibuprofen or aspirin during pregnancy, these drugs are still considered as generally safe and are recommended by doctors with certain considerations (Hurtado-Gonzalez & Mitchell, 2017; Kristensen et al., 2016).

One of the major problems with studying the effects of analgesics during pregnancy is the difficulty of direct studies in humans. A big proportion of the research done is epidemiological (Snijder et al., 2012; Sommese et al., 2017) and many others have used rodent models (Dean et al., 2016; Holm et al., 2015; Kristensen et al., 2012), with the adjacent problems of these types of studies. More recent studies have used human fetal gonads from abortuses to perform experiments in vitro (Mazaud-Guittot et al., 2013) or in xenografts in nude mice (van den Driesche et al., 2015). Despite the similar results among many of these studies, not all of the outcomes correlate within all of the publications and there are important discrepancies, especially among times and length of exposure. The discrepancies in the literature and the inherent difficulties in translating experimental research findings into robust evidence for action with respect to human pregnancy demand an approach involving different

models. In this thesis, I have combined research using different species: rat and human in both, males and females; but also involving different approaches, such as in vivo analysis (rat only) and in vitro studies using fetal gonad hanging drop (rat and human) and human fetal testes xenografted into mice. Furthermore, I have also used a cellular model to allow deeper study of the mechanistic pathways modified by analgesics.

Diverse studies have shown a link between fetal analgesic exposure and development of male reproductive disorders (cryptorchidism or hypospadias) (Kristensen et al., 2011; Snijder et al., 2012), which are known to be related to altered testosterone exposure and Leydig cell function (Kristensen et al., 2011; Mazaud-Guittot et al., 2013; van den Driesche et al., 2015). Few studies focused on alterations in normal GC development and consequent effects on fertility, but only in rodents (Dean et al., 2016; Holm et al., 2015; 2016). The use of human fetal samples in order to study the effect of analgesics on GC was analysed for first time in this thesis using the hanging drop culture method and the xenograft model (Chapter 4).

The mechanisms of action of analgesics in the fetal gonads has not been well studied. The study of the mechanisms of action of analgesics is important because by understanding these mechanisms, we are better placed to prevent adverse effects and to exploit any beneficial effects. A possible common mechanistic pathway that might link analgesics and the phenotypes seen, is the PGE_2 pathway. Somatic and germ cells in the fetal gonads of both sexes in rats and humans are sources and targets of PGE2 as they express the PG-synthesizing COX2 enzyme and PGE receptors. Alterations in this pathway have been linked with modifications in cell proliferation (Kimura et al., 2001; Krause et al., 2007; Mendez & LaPointe, 2005; Mo et al., 2015; Sanchez & Moreno, 2002), mECS pluripotency (Yun et al., 2009) and cell epigenome (Arosh et al., 2015; Huang et al., 2012; Xia et al., 2012). Furthermore, during the time of my thesis, another project from my lab (now published), found that analgesics

were able to affect not only the exposed generation (F1), but also showed inter-generational consequences, affecting the F2 generation that resulted from mating the F1 generation (males or females) with wild-type rats (Dean et al., 2016). In this thesis I performed a deeper analysis on the possible mechanisms altered by analgesics in GC. First, in different in vitro and in vivo rat models (chapter 3). Second and in the context of human fetal GC, mechanistic studies were performed with NT2 cells, a human GC cellular model (Chapter 5).

1.6.1 Hypotheses

- Paracetamol and Ibuprofen are able to alter mechanisms that affect normal fetal GC development.
- In vitro models can be used for the study of the effects of analgesic exposure on fetal gonad GCs.
- Analgesic can affect proliferation, the pluripotency states and the epigenetic machinery of GCs.
- Analgesic modifications in fetal gonad are mediated by the prostaglandin E2 pathway.

1.6.2 Aims

- Develop in vitro fetal testis and ovary culture methods for human and rat, to explore the effects of analgesics/PGs on fetal GC.
- To study the consequences of analgesics exposure on human fetal testicular GCs in a xenograft model.
- Evaluate whether analgesic exposure of the fetus causes epigenetic changes to GC that might affect the next/future generations.

- To investigate the possible involvement of prostaglandin E2 as a local mediator of analgesics effects on the fetal gonad.

Chapter 2: Material and methods

2.1. Animal work

All aspects of animal housing, management and treatment was undertaken in the Little France Biomedical Research Facility (BRF) according to the UK Home Office Animal Experimentation Scientific Procedures act 1986, under the project licence RMS - PPL 60/4564. Wistar rats were obtained from Harlam UK and bred within the facility to generate stock that were used for experiments. William Mungal undertook the daily husbandry. I conducted procedures under my personal license I2F5A0009.

2.1.1. Welfare conditions

Animals were housed in the animal facility under fixed conditions of light (12h per day from 07:00 to 19:00), humidity (55%) and temperature (20-25ºC). Rats were housed under standard conditions and had free access to tap water and a soy-free diet (SDS; Dundee, Scotland). Rats were kept in clear-sided polypropylene cages, containing solid bottoms covered in wood shaving for bedding.

2.1.2. Time-mating

Timed-matings were used to determine gestational age. Sexually mature males (aged between 10 and 14 weeks) and females (over 10 weeks old) were kept separated except for mating purposes, when they were paired together 1:1 in a grid-bottomed cage overnight. Time-mating was established by the presence of a vaginal plug in the morning, indicating a successful mating and the gestational age was thus recorded as embryonic day 0.5 (e0.5). The male was separated from the female after mating and pregnant females were then housed separately.

2.1.3. Treatment during pregnancy

Few studies have focused on how maternal paracetamol exposure can affect gene expression during fetal gonad development. To address this, pregnant female rats were administered paracetamol or indomethacin. Paracetamol (350 mg/kg/day) was suspended in corn oil and administered via oral gavage using a 15-16G blunt ended

steel gavage cannula, 10-12 cm in length (Medicut, Sherwood Medical Industries Ltd, UK) attached to a disposable plastic 1mL syringe. The paracetamol suspension was prepared in advance and stored at room temperature prior to use. Indomethacin (0.8mg/kg) was administered by subcutaneous injection in corn oil. This concentration was used following previous studies (Dean et al., 2013), which showed that higher doses, which have been used in mice (Gupta & Goldman, 1986), resulted in litter loss and internal bleeding. To ensure a correct drug dose, rats were weighed directly before each daily treatment. Treatment was administered in the morning (between 09:00 and 11:00). Animals were checked regularly for any sign of discomfort or toxicity during treatment. Paracetamol was administered from e13.5 until the day before sampling (e15.5 or e17.5), as described previously (Dean et al., 2016; Dean et al., 2013). This time-period is part of the MPW, where testosterone production is crucial for setting up later masculinization (Welsh et al., 2008). Control dams were administered corn oil according to the same regimen (Figure 2.1).

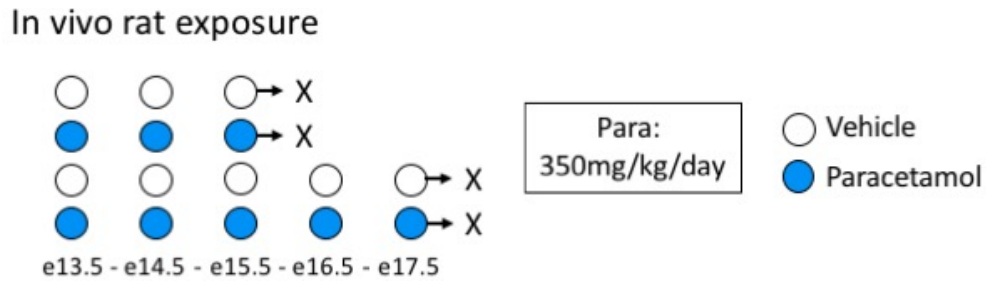


Figure 2.1. Representation of in vivo rat exposure regimes to vehicle or paracetamol

2.1.4. Necropsy

After treatment, pregnant rats were killed by cervical dislocation after increasing inhalation of carbon dioxide (CO_2), under schedule 1 of the animal (scientific procedures) Act 1986. The uterus was then removed by dissection and placed on ice.

2.1.5. Gonad dissection

The uterus was opened with the help of dissecting scissors and an average of 8-15 fetuses were recovered from each uterus. Fetuses were maintained in ice-cold 0.01M phosphate buffered saline (PBS; Sigma) in order to minimise degradation. Each fetus was placed in a petri dish (Corning) containing PBS and its abdomen opened under a binocular dissecting microscope (Leica, MZ6). Extra lighting was provided by external cold lights (Leica CLS 150x) in order to minimise temperature rise. Gonads were excised using the bevelled edge of 27G needles (Monoject, sterile needles, 0.4mm x 12mm) attached to disposable plastic 1ml syringes. These, and a scalpel (Swann-Morton) were then used to remove the epididymis and the mesonephros from the fetal gonads. Sander Van den Driesche trained me and helped me with gonad dissection. Sex was determined by gross morphological differences between testes and ovaries. Thus, the larger size, striped appearance (seminiferous cords) and testis-specific vasculature indicated a male fetus, whereas absence of these features was indicative of a female fetus.

2.1.6. Tissue preservation

Samples were immediately preserved after their retrieval. Depending on the use for future experiments, they were or either snap-frozen on dry ice or stored in Bouin´s solution. Frozen samples were stored at -80ºC for long term preservation for future use in RNA expression analysis. In this case, and in order to obtain enough RNA, both ovaries were frozen together in one 2mL Eppendorf tube, while only one testis was needed for that purpose. Fixation in Bouin´s solution (Clin-tech) was performed for 2 hours before submerging the gonads in 70% ethanol for storage prior to their processing and embedding as below.

2.2. Rat gonad culture

In order to establish if there were direct effects of the analgesics on the fetal gonad and thus to separate them from possible indirect effects caused by the maternal exposure, fetal rat gonads were cultured in vitro in the presence of paracetamol or ibuprofen. Moreover, further studies using prostaglandin receptor (EP2+EP4) antagonists were performed in order to determine the relationship between the analgesics pathway of action and the prostaglandin E2 pathway.

2.2.1. Hanging drop culture

Rat fetal (e15.5) testes and ovaries were dissected and cut into ~1mm^3 pieces (n=13-30). Single pieces were cultured in hanging drops containing 30µL culture medium (Alpha-MEM; Lonza) supplemented with 10% fetal bovine serum (Life Technologies), 10µL/mL penicillin/streptomycin (Sigma-Aldrich), 1% 200mM L-glutamine (100X) (Gibco), 1% MEM NEAA non-essential amino acids (100X) (Gibco), 2% sodium pyruvate 100mM (100X) (Gibco) and 1% Insulin-transferrin-Selenium (100x) (Gibco). Media was freshly prepared at the beginning of each experiment. Several tissue pieces from the same gonad were placed in the same petri dish and incubated for 48h at 37ºC under 5% CO_2. At the end of the culture period, tissue samples were fixed in Bouins for 2h for subsequent analysis by fluorescence immunohistochemistry, or snap-frozen for qPCR analysis. (Figure 2.2)

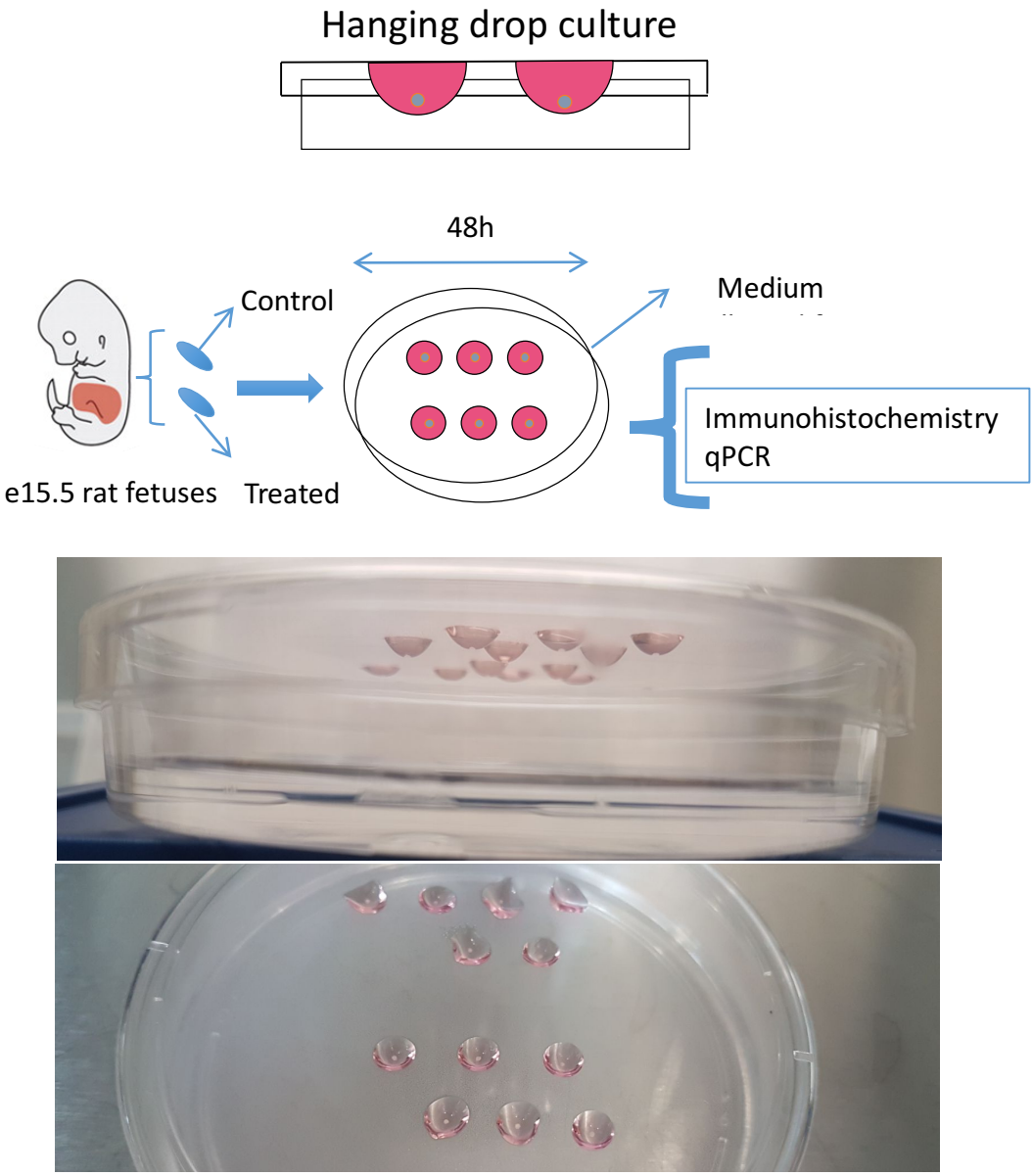


Figure 2.2. Rat fetal gonad culture in hanging drop. A. schematic representation of the rat fetal gonad hanging drop model system. **B** Hanging drop culture. 30µl medium drops, in which the fetal gonad pieces were placed, are distributed on the underside of the cover of a petri dish, which contains PBS.

2.2.2. Treatments

Tissues were cultured in medium to which was added vehicle, paracetamol (10µM), ibuprofen (10µM; Sigma-Aldrich) or L-161,982 (EP2 antagonist; 10µM; Cayman chemical) + PF0441848 (EP4 antagonist; 10µM; Cayman chemical). Drugs were diluted in dimethyl sulfoxide (DMSO) to a final concentration of 10mM and kept at -20ºC for long term storage. The analgesic doses used reflect the lower end of the range of serum concentrations in humans following therapeutic analgesic exposure (10µM), which are relevant to peak serum concentration found in humans after therapeutic exposure (1.37 µM in the case of paracetamol 48h after exposure and 2.5 10µM with ibuprofen 1h after exposure) (Mazaud-Guittot et al., 2013; Kristensen et al., 2012; Janssen & Venema, 1985). (Figure 2.3).

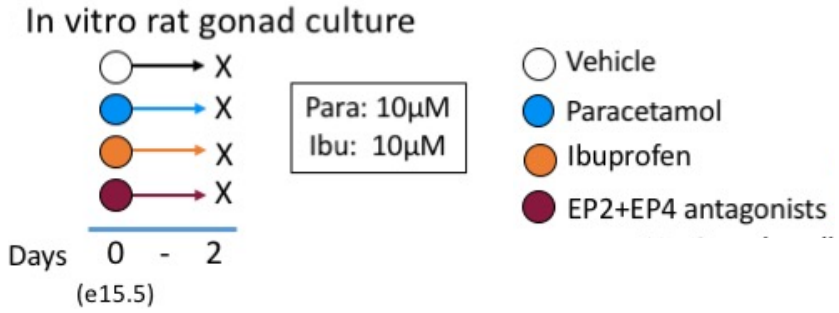


Figure 2.3. Representation of in vitro rat gonad culture exposure regimes. Rat gonads were cultured with different treatments (Vehicle - white; Paracetamol - blue - 10 µM; Ibuprofen - orange - 10 µM; EP2+EP4 antagonists - maroon, 10 µM each) for 48h.

2.3. Human fetal tissue collection

Further analyses were undertaken on human fetal gonadal tissue. Human fetuses were obtained following termination of pregnancy in different research facilities: Royal infirmary hospital in Edinburgh, and from the Human Developmental Biology Resource (Newcastle and and London). All women gave written consent following the UK national guidelines (Polkinghorne 1989) and the use of the fetal tissue was approved by the respective Local Research Ethics Committee (reference number LREC08/S1101/1 for Edinburgh). For the induction of termination, women were administered mifepristone (200mg orally). Depending on the week of gestation,

women were also administered misoprostol (Pharmacia, Surrey UK 200mg every 3h, per vaginam) or a vacuum aspiration was performed under general anaesthetic. Samples from terminations for fetal abnormality were not used for this study. Fetal gestational age was calculated by ultrasound and foot length (2nd trimester only). The extraction of the fetal gonads was performed by the hospital team when samples came from Newcastle or London and sent on ice to Edinburgh for immediate experiments. Samples from the Edinburgh unit were dissected by Rosemary Bayne and Roseanne Rosario. The extraction was performed by micro-dissection from the fetal abdominal cavity. 1st trimester fetal gonads (testis and ovaries) were cultured using the hanging drop culture method. 2nd trimester fetal testes were xenografted into nude mice as described below. A small piece of each gonadal sample was fixed in Bouin's as pre-graft/pre-culture controls.

2.3.1. Sex determination

Sex was determined by morphology when possible (2nd trimester only) or, for 1st trimester samples, by qPCR to detect the presence of the male SRY gene. When possible, qPCR was performed using skin tissue. This tissue was digested in 100µL of digestion buffer (25mM NaOH/0.2mM EDTA; Sigma) for 20 minutes at 95ºC. Digestion was stopped by adding 100µL of neutralization buffer (40mM Tris-HCL, Sigma) before being vigorously vortexed and being stored at -20ºC. Sex determination was analysedby the expression of SRY using the SYBR green method. The process of qPCR is explained in detail below (Section 2.8.5). The reaction was performed using a FAST SYBR® qPRC kit (Kapa Biosystems), according to the manufacturer´s instructions (Table 2.1). A master mix was prepared by mixing qPCR Master Mix (2x) universal and the specific forward and reverse primers, following the instructions' recipe and 9µL of the prepared master mix was added to each well, as well as 1µL of the sample. Positive and two negative controls (female and water only) were added to each run. The melting curves produced by the program run (Table 2.1)

were then analyzed in order to see the presence or absence of a peak, typically seen with the presence of SRY (Figure 2.4)

Table 2.1. SYBR green qPCR. Regents required for a qPCR reaction using SYBR green kit (Up). Temperature and timing required for a qPCR reaction using SYBR green kit (Down).

Reaction	Volume per tube (20 µL)	
qPCR Master Mix (2x) Universal	8.4	
Forward primer (5'-ATCTGCGGGAAGCAAACTGC-3')	0.3	
Reverse primer (5'-ACAGTAAAGGCAACGTCCAG-3')	0.3	
Sample	1	
Temperature (ºC)	**Time (seconds)**	
95	180	
95	3	X 40 cycles
60	20	
95	5	Melting curve
65	60	
97	10	

Negative (water) Female sample Male sample

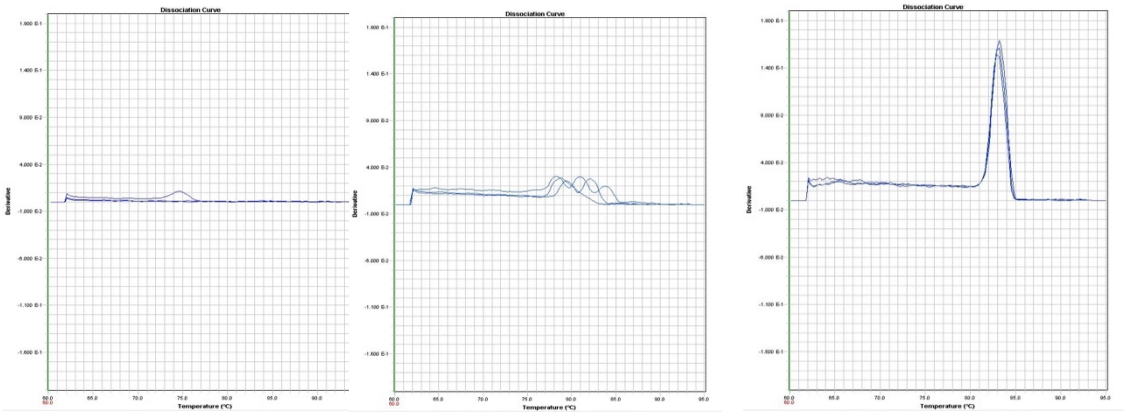


Figure 2.4. Examples of qPCR analysis for sex determination. The figure shows examples for a negative reaction (left), a female sample (centre) and a male sample (right).

2.3.2. Human hanging drop cultures

1st trimester human fetal testes (n=4; 8-11 gestational weeks) and ovaries (n=3 9-11 gestational weeks) were cultured following a similar protocol as described for rat fetal gonad cultures. This protocol was adapted for human cultures, increasing the culturing time and adding hCG to the medium in order to mimic the in-utero environment. Our lab found that the presence of hCG in the medium partially prevented the culture-induce GC loss, showing higher number of GC present in the samples after the cultures. For that reason, all human fetal gonad cultures were supplemented with hCG. After culture, samples were fixed for 90 mins in Bouin´s and the culture medium from each day was frozen each day after pooling the medium from the replicate pieces from the same fetus and treatment.

2.3.2.1. Human hanging drop treatments

Plates were incubated for 8 days at 37ºC under 5% CO_2 and the medium was changed every day. Samples were cultured with hCG alone during the first day, and thereafter the medium was supplemented with hCG plus vehicle or analgesic (10µM paracetamol or 10µM ibuprofen) for the remainder of the culture period (Figure 2.5).

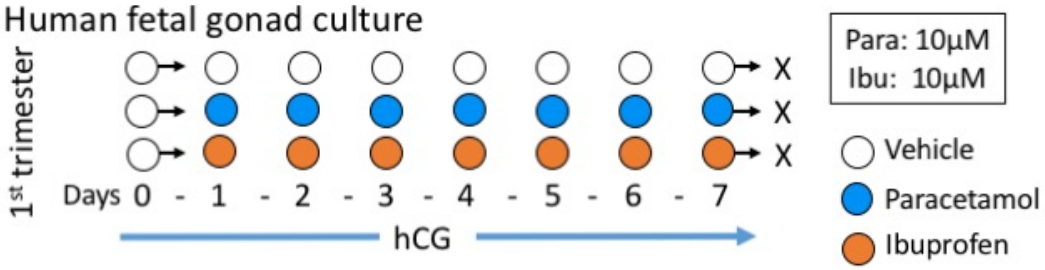


Figure 2.5. Representation of in vitro human gonad culture exposure regimes. Human gonads were exposed to different treatments (Vehicle - white; Paracetamol - blue - 10 µM; Ibuprofen - orange - 10 µM) for 7 days. Samples were also exposed to hCG during the exposure. Treatment was replaced every 24h.

2.3.3. Xenografts

2nd trimester human fetal testis tissue was used for ex-vivo xenograft studies. The 2nd trimester samples are bigger in size than 1st trimester, providing enough material for xenografting it. This system offers a more physiological model in which to study gonad development than hanging drop cultures (Mitchell et al., 2010). Ovary xenografts were attempted previously by other members of the lab, but they did not achieve good viability, and therefore xenograft experiments were only performed with testis tissue. Male CD1 nude (host) mice (aged 4-6 weeks; n =44; Charles River UK) were used for these studies. Anaesthesia by isoflurane inhalation was provided to the host mice before they were castrated through a scrotal incision at least 2 weeks before the xenograft process. Further analgesia (carprofen, Pfizer) was supplemented in the drinking water for 3 days after the castration procedure.

Fetal testes (N=8; 14-17 gestational weeks) were dissected and placed immediately into ice-cold medium containing Leibovitz's L-15 (Sigma-Aldrich) with glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% non- essential amino acids. Samples were cut into ~1mm^3 pieces and xenografted into castrated nude mice as described previously (van den Driesche et al., 2015). An average of 4-6 pieces of gonadal tissue were inserted subcutaneously under the dorsal skin of each host on either side of the dorsal midline with the help of a 13-gauge cancer implant needle (Popper and Sons). Mice were randomly allocated to receive the different treatments. Grafting and extraction were performed by Joni Macdonald and mouse treatments were administered by William Mungal.

2.3.3.1. Treatments of xenografted mice

Grafts were allowed to grow in the host for a total of 14 days. The first 7 days allowed the establishment of a blood supply to the xenografts. This was followed by daily paracetamol or ibuprofen treatment, for a period of 1 (paracetamol only) or 7 days. For the 1 day paracetamol treatment, mouse hosts received vehicle treatment for 6 extra days. In order to create a more in-utero environment, host mice received an

additional 20 IU hCG treatment (Pregnyl, Organon Laboratories) by subcutaneous injections every 72h (van den Driesche et al., 2015). Host mice were administered either vehicle (corn oil), paracetamol (20mg/kg, three times daily) or ibuprofen (10mg/kg, three times daily); both analgesics were suspended in corn oil and administered by gavage. Host mice were sacrificed by cervical dislocation and grafts were retrieved and weighed before fixation in Bouins. Sections of this fixed tissue were subsequently used for fluorescence immunohistochemistry. (Figure 2.6)

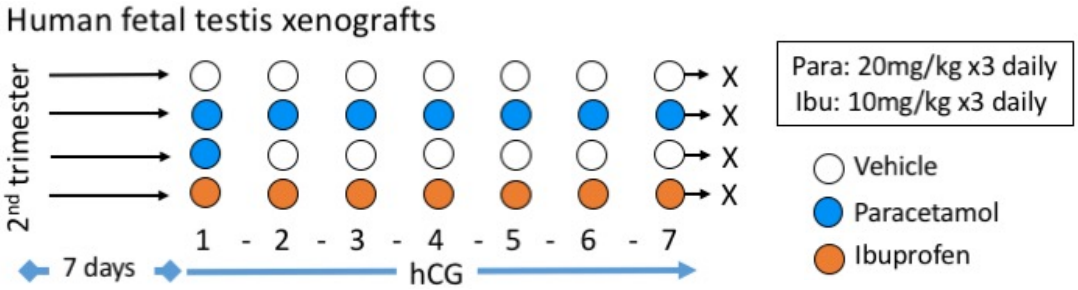


Figure 2.6. Representation of in vivo human gonad culture exposure regimes. Mice were exposed to different treatments (Vehicle - white; Paracetamol - blue - 10 µM; Ibuprofen - orange - 10 µM) for 7 days. Samples were xenografted for 7 days before the start of the treatment. Mice were also exposed to hCG during the experiment. Treatment was given every 24h and samples collected 2h after the last exposure.

2.4. Protein detection by fluorescence immunohistochemistry

Immunohistochemistry was used to determine the expression of specific proteins in the different gonadal cell types and to compare between treatments. It also enabled accurate counting of the different cell populations. Only fluorescence immunohistochemistry was used in the experiments for this thesis. This technique is based on the capacity of antibodies to detect specific peptides, which can be targeted to detect a fragment of a protein of interest. These antibodies are referred to as primary antibodies. For signal amplification purposes, secondary antibodies raised against the species of the primary antibody were used. These secondary antibodies are attached to horseradish peroxidase (HRP), which allows indirect detection. For non-fluorescence immunohistochemistry, HRP is normally used in combination with

a substrate that will be able to produce a colour substance that stains the places where the protein of interest is localised. In this case, HRP was used in combination with a fluorescent Tyramide signal amplification. Tyramide is a compound able to bind to electron rich moieties on the surface of the tissue, but only when it is adjacent to the HRP. Tyramide is conjugated with a fluorophore, enabling rapid and sensitive fluorescence amplification (Figure 2.7).

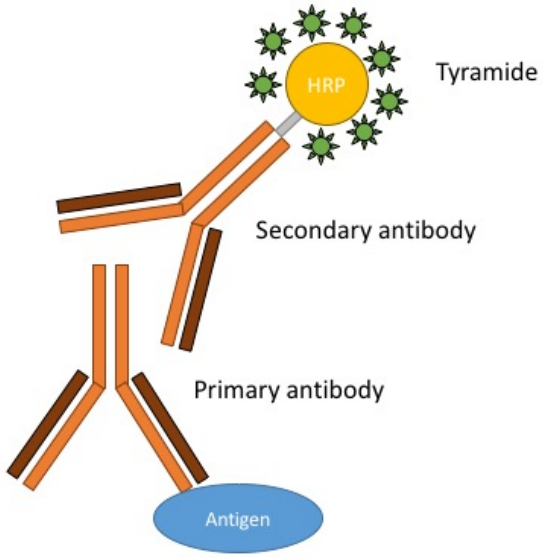


Figure 2.7. Fluorescence immunohistochemistry detection. Schematic representation of fluorescence immunohistochemistry detection of proteins. The antigen of interest is bound by the primary antibody. This primary antibody is then detected by the secondary antibody, which is attached to horseradish peroxidase molecule. This last molecule catalyses a reaction between the surface of the tissue and the Tyramide, which is conjugated to a fluoresphore, detectable by confocal microscopy.

2.4.1. Fixation

Once the tissue was collected, it was fixed in Bouin´s solution (20:5:1 picric acid, formalin, acetic acid; Clintech). The duration of the fixation was dependant on the size of the sample. As a general rule, rat fetal whole gonads were fixed for a total of

4 hours and human gonad pieces were fixed for 2 hours. Once the tissue was fixed, the Bouin´s solution was discarded and replaced with 70% (v/v) ethanol. Samples were then stored until ready for paraffin embedding.

2.4.2. Paraffin embedding

After Bouin´s fixation, samples were embedded in paraffin by the histology team of the small university research facility (SURF) at the university of Edinburgh. Tissue was submerged in a number of different concentrations of alcohol (70-100%) overnight in an automated Leica ASP300S tissue processor (Leica microsystems). Subsequently, samples were placed manually into blocks of molten paraffin wax with the help of a histoStar (Thermo Scientific). When cold, tissue blocks were stored at room temperature for future use.

2.4.3. Sectioning

Tissue samples embedded in paraffin blocks were cut into sections using a microtome (Leica#RM212 5RT). Blocks were cooled on ice for at least 30 minutes before cutting in order to allow a cleaner cut. Serial sections (5µm) were obtained from the blocks, which were placed into 30% (v/v) industrial methylated spirits (IMS; Fischer Scientific) before placing them onto a 45ºC water bath (Lamb) to allow creases to unfold. Sections were then separated with the help of fine brushes and mounted onto coated slides (BDH Chemicals). Slides were kept at 45ºC during cutting in a heater (Lamb) and at 55ºC overnight in order to dry the sections and allow attachment to the slides. Afterwards, slides were cooled to room temperature for long term storage.

2.4.4. Dewaxing and rehydrating

In order to prepare the tissue for immunochemistry, slides need to be dewaxed and tissue rehydrated. Paraffin wax needs to be removed from the sections by a double incubation in xylene (VWR chemicals) for 5 minutes. Rehydration was performed by

submerging slides in a decreasing graded ethanol series (VWR Chemicals) (v/v) (100%, 100%, 95%, 80%, 70%) for 20 seconds each before rinsing in tap water.

2.4.5. Hematoxilyn and eosin stain

Some slides were stained following the hematoxilyn and eosin (H&E) staining method. Using this technique, a combination of hematoxilyn and eosin reagents are used to stain acidic (blue) and basic (red/pink) substances respectively. This allows us to differentiate the structure and morphology of the tissue. For this method and after the rehydration of the tissue, the slides were submerged in hematoxilyn for 5 minutes. After a wash, they were treated with acid alcohol to remove the excess of hematoxilyn. Samples are then washed and submerged in Scott´s tap water solution (STWS) for 30 seconds before being washed again and submerged in Eosin for 10 seconds and washed. After these steps, the samples were already stained and they needed to be dehydrated again with increasing graded ethanol series (VWR Chemicals) (v/v) (70%, 80%, 95%, 100%, 100%) for 20 seconds each before rinsing in tap water.

2.4.6. Antigen retrieval

During Bouin´s fixation, proteins undergo cross linking, which improves the tissue integrity, but which may hide the detectable antigens. Antigen retrieval allows these antigens to be exposed again to the antibodies. To achieve this, sections were pressure-cooked in 0.01M citrate buffer (pH 6.0). The decloaking chamber (Biocare Medical) was programmed to bring slides to 125ºC for 30 seconds, and sections remained inside the chamber for a further 20 minutes after it cooled to 90^{o}C, so the slides can be cooled to room temperature with tap water.

2.4.7. Blocking

Slides were immediately incubated in methanol (Fischer,) with 3% (v/v) hydrogen peroxide (VWR chemicals) for 30 minutes at room temperature on a rocking

platform, in order to eliminate any residual enzymatic activity that could potentially interfere with the immunohistochemistry process. Slides were then washed once in tap water and again in Tris-buffered saline [TBS: 0.05 m Tris, 0.85% NaCl (pH 7.4)] for 5 minutes at room temperature on a rocking platform. Samples were then dried carefully to remove the TBS but so as not to disrupt the tissue, and then the sections were incubated in a humidified chamber for 30 min with 20% chicken serum, 5% bovine serum albumin (BSA) (Sigma) in TBS to block non-specific binding of the antibodies.

2.4.8. Primary antibody

Each primary antibody used was first optimized for the different tissues, species and sexes. For the optimization, different concentrations of the antibody were used and final results were compared in order to select the conditions giving the best outcomes. The primary antibody was then diluted in the chicken serum. Blocking buffer on the slides was removed before adding the primary antibody, which was incubated at 4ºC overnight in a humidified chamber. As negative controls, one slide was treated with blocking solution (no antibody) overnight. The list of the different antibodies used and their concentrations are shown in Table 2.2.

2.4.9. Secondary antibody

Tissue was then incubated with the appropriate secondary antibody, which was raised in the same species as the blocking serum and against a species-specific sequence on the primary antibody. Secondary antibodies are conjugated to HRP (Santa Cruz Biotechnology), which will later allow the attachment of the fluorophore conjugated Tyramide. After incubation with the primary antibody, tissue was washed twice in TBS (5 minutes each) for the removal of unbound primary antibody. This step was then followed by incubation with the secondary antibody at a concentration of 1:200 in TBS (Table 2.2). Slides were incubated for 30 min at room temperature in a humidified chamber.

Table 2.2. List of different antibodies used for immunochemistry experiments.

Antibody	Producer	Raised in	Raised against	Cat No	Dilution	Purpose
Aldh1a1	Abcam	Rabbit	Rat	Ab23375	1:700	Expression
β-catenin	Abcam	Rabbit	Rat	ab32572	1:2000	Localization
Sp1	Abcam	Rabbit	Rat	ab13370	1:1000	Localization
SOX9	Millipore	Rabbit	Human	AB5535	1:5000	Cell count
AP2γ	SantaCruz	Mouse	Human	sc-12762	1:75	Cell count
MAGE-A4	Gift-Giulio Spagnoli	Mouse	Human	-	1:300	Cell count
Ki-67	Abcam	Rabbit	Human	ab16667	1:100	Cell count
SytoxGreen	MolecularProbes	-	-	S7020	1:1000	Counter stain
Hoechst	Thermo Fisher	-	-	H1399	1:4000	Counter stain
Secondary-Peroxidase	SantaCruz	Chicken	Rabbit	Sc-2963	1:200	Staining
Secondary-Peroxidase	SantaCruz	Chicken	Mouse	Sc-2954	1:200	Staining

2.4.10. Tyramide

Immediately after the secondary antibody incubation, slides were washed twice in TBS at room temperature on the rocking platform. Sections were then incubated for 10 min with Tyramide Signal Amplification (TSA Plus Cyanine 3 System, PerkinElmer Life Sciences), conjugated with the appropriate fluorophore and diluted 1:50 in the diluent provided by the manufacturer. The reaction was stop by washing the slides in TBS twice at room temperature.

2.4.11. Multiplexed assays

The Tyramide technology allows the visualization of different antigens by different antibodies on the same tissue, even if the antibodies are raised in the same species. If primary antibodies are raised in a different species, slides were incubated in 3% hydrogen peroxide in TBS-triton (TBST-T; Sigma) for 15 minutes at room temperature in the dark. This was followed by two TBS washes at room temperature on a rocking platform in the dark as well. If primary antibodies derived from the same species, for each subsequent primary antibody, sections underwent further antigen retrieval by boiling in 0.01M citrate buffer (pH 6.0) for 2 min in a decloaking chamber, allowing the slides to cool for 20 minutes before washing the slides in TBS twice at room temperature on a rocking platform in the dark. This allowed the primary and secondary antibodies to be removed from the tissue, but maintained the Tyramide signal. After one of the last two options, this was followed by blocking with chicken serum, overnight incubation with the primary antibody, incubation with the appropriate secondary antibody and Tyramide as described above.

2.4.12. Nuclear counterstain

In some cases, and to help with visualisation of tissue architecture, a nuclear counterstain was performed as well. After the Tyramide step, and following washes, slides were exposed to a nuclear counterstain, SytoxGreen (Molecular Probes) or

Hoechst (Thermo Fisher), diluted in TBS, for 10 minutes at room temperature and in a dark humidified chamber. Counterstain concentrations are described in Table 2.2

2.4.13. Coverslip mounting

Slides were washed in TBS and mounted in aqueous mounting medium (Permafluor; Beckman Coulter, High Wycombe), covered by a glass coverslip (Leica). Slides were then dried by hand around the borders, checked for bubbles and wrapped in foil paper before storage in a darkroom at 4ºC until imaging was performed.

2.4.14. Imaging

The localization of the proteins of interest was examined using a Axiovision LSM 710 or 780 confocal microscopes (Zeiss) and using Zen software (Zeiss)**.** For human gonad studies, whole tissue images were taken as tiles with a 10% overlap, following the software instructions. Images from H&E staining were obtained using a Provis microscope (Olympus Optical) and images were taken with a fitted DCS330 digital camera (Eastman Kodak). Treatment of images for this thesis was performed using Adobe Photosohop 5.0 (Adobe Systems Inc).

2.5. Image analysis

2.5.1. Determination of rat fetal gonad culture development

To analyse and validate the rat fetal gonad cultures, culture viability and gonadal development were compared with a corresponding age-matched (non-cultured) control sample. To do so, rat testis and ovary culture tissue were processed as explained above, and then immunostained for Vasa, to determine the presence of GC, and for Sox9, in order to study the presence of Sertoli cells (male gonads only). Moreover, to study fetal gonad culture development, two other antigens were evaluated: Dmrt1, for ovary development, and Oct4 for testis development. Both proteins are expressed in e15.5 rat gonads, but their expression is highly reduced or

absent by e17.5. Therefore, comparison was made between cultured (e15.5 gonads cultured for 48h) and age-matched controls (e15.5 and e17.5).

2.5.2. Quantification of GC number in human fetal gonads

As variably-sized pieces of whole testicular and ovarian fetal tissue were used for culture or xenografting, GC number was determined in relation to the tissue section area (ovaries) or to seminiferous cord area (1st trimester testes). The delineated structures containing Sertoli cells (SOX9^{+}) were defined as the seminiferous cord area. In the case of 2nd trimester fetal testes, seminiferous cord area was not calculated. Instead, GC number was expressed relative to the major somatic cell component of the seminiferous cords, in this case, Sertoli cells (i.e. number of SOX9+ cells).

One randomly selected fetal testis section per cultured or xenografted tissue piece was co-immunostained, following the same protocol explained above, using antibodies for SOX9 (Sertoli), AP2γ (pluripotent gonocytes) and MAGE-A4 (non-pluripotent pre-spermatogonia). Blinded to treatment, I then counted (using ZEN software) the total number of cells from the different cell populations, as well as the seminiferous cord or ovary area. After all the slides were processed, treatments were revealed and statistical calculations were performed. The total number of AP2γ^{+} and MAGE-A4^{+} GC per section was expressed relative to the total area of the seminiferous cords in that section. Three different ratios were determined: Total GC (AP2γ^{+} + MAGE-A4^{+}), AP2γ^{+} GC and MAGE-A4^{+} GC per μm^2. In the case of the xenograft experiments for 2nd trimester fetal testes, the number of GC was expressed relative to the total number of Sertoli cells (SOX9^{+}) in each section**.** Three further ratios were then determined: Total GC (AP2γ^{+} + MAGE-A4^{+}) per Sertoli cell and AP2γ^{+} GC per Sertoli cell and MAGE-A4^{+} GC per Sertoli cell.

Fetal ovary sections (1 per cultured piece) were co-immunostained using antibodies and AP2γ (to identify all the GC) and Hoechst (Thermofisher) as a counterstain. Mage-A4 is not expressed in ovarian GC and, therefore, it was not used in the ovary samples. Similarly to the testis analysis, the total number of AP2γ^+ GC per section was quantified and expressed relative to the total tissue section area. The ratio of AP2γ^+ GC per μm^2 was calculated and compared between vehicle and treatment groups.

2.5.3. **Quantification of GC proliferation in human fetal gonads**

For our experiments, I also determined GC proliferation. For this purpose, testicular and ovarian sections were immunostained for AP2γ, MAGE-A4 (testis only), as explained above, but with the addition of Ki-67, a known marker of proliferation (Scholzen & Gerdes 2000). Proliferative GC were defined as cells expressing any GC marker (AP2γ or MAGE-A4), as well as Ki67. Proliferative GC were quantified for total GC (AP2γ^+ or MAGE-A4$^+$), AP2γ^+ GC and MAGE-A4$^+$ GC as described above.

2.6. NT2 cell research

In view of the effects of analgesic exposure on GC number, it was decided to use a more tractable system, in this case a cellular model, to study in deeper detail the possible effects of analgesics on different cellular mechanisms. Currently it is not possible to culture fetal germ cells in vitro, due to difficulties in maintaining cellular division and growth. For that reason, the NT2 cell model was chosen, as it shows similar characteristics to human fetal GC, but with a consistent and fast cell division.

NT2 are a pluripotent human embryonal carcinoma cell line, which derives from a metastatic testicular GC tumor, originally produced in 1984 from a male patient. They were collected from the lung of the patient and xenografted in mice before the final extraction and process of becoming an established cell line. During their original extraction, different analyses were performed to confirm their embryonal carcinoma

origin. They exhibit the morphology and cell surface antigens of human embryonal carcinoma cells, such as a high nuclear-cytoplasmic ratio, prominent nucleoli and expression of the antigen SSEA-3 (Andrews et al., 1984).

As a tumoural cell line, they have a fast and consistent cell cycle, being able to duplicate their cell number in 48h. GC tumors are believed to derive from arrested development of fetal pluripotent GC, and accordingly, they show similar characteristics, such as the expression of fetal GC markers, such as AP2γ and hence they are commonly used as a fetal GC model (Hoei-Hansen et al., 2004).

2.6.1. Cell culture settings

NT2 cells were obtained as a gift from Anne Jorgensen in Copenhagen. After an original expansion, vials containing 1 million cells on passage 23, suspended in 1mL Bambaker solution (Nippon Genetics) were stored at -80ºC during the time the present experiments performed. A new vial was thawed and expanded for each new set of experiments, so each set was started with cells from the same original vial. Experiments were carried out inside a sterile hood (BioMat^2; Medical Air Technologies), regularly decontaminated with H_20_2 vaporization. Hoods were carefully cleaned and decontaminated on a regular 3-month basis. Before every experiment, the hood was also decontaminated with 30 min ultraviolet light followed by a 70% ethanol disinfection. Cells were regularly checked for mycoplasma infections via Elisa using an internal service provided by Forbes Howie. Cells were maintained in an incubator (Hera cell; Heareus) containing water to maintain high humidity. The incubator was regularly decontaminated by 70% ethanol followed by a decontamination routine according to the manufacturer's protocol. This decontamination routine consisted of four different phases: a heating phase, where the space was heated to 90^{o}C with elevated humidity for 2 hours; a decontamination phase, which lasted 9 hours and during which the temperature was kept high; a cool-down phase, in which the device cooled down to 37^{o}C again for 9 hours; and finally,

a post-heating phase, in which condensation inside the incubator was eliminated for the last 3 hours. The incubator was kept at 37^{o}C and 5% CO2 as normal conditions for cell culture.

2.6.2. Cell thaw

Vials were taken out from the -80ºC and kept on dry ice. Vials were thawed in a warm water bath for around 1-2 minutes for a fast melt and quickly transferred into a sterile hood, where 1mL of pre-warmed medium (D-mem; Gibco) supplemented with 10% FBS, 10µL/mL penicillin/streptomycin and 1% 200mM L-glutamine, was carefully added to the cells. The suspension was then transferred into a 2mL Eppendorf tube to be centrifuged. Cells were spun down at 4000 rpm for 5 min. The supernatant was then discarded and cells were resuspended in 1 mL medium, which was transferred to a T75 filtered cap (Corning) filled with 13 mL warmed medium.

2.6.3. Cell maintenance and expansion

Cells were maintained in T75 filtered caps until 80-90% confluence was reached, before being split. Cell confluence was regularly checked using a cell microscope (CK40; Olympus). For splitting, medium was discarded and the cells washed with pre-warmed 1x PBS, and disaggregated with 3mL TrypLE™ Express (Gibco) for 5 minutes at 37ºC. TrypLE is a highly purified cell-disassociation enzyme that replaces the traditional use of trypsin and is able to disassociate attachment-dependent cell lines. After cells were detached, the enzymatic activity of TrypLE was stopped by adding 7mL pre-warmed medium. Cells were then transferred to another T75 flask containing medium in a ratio 1:2 or 1:4. This process was also used for cell expansion after the vial was thawed to get enough numbers of cells for the future experiments.

2.6.4. Cell quantification

For seeding or treatment effects, cell number was determined using a NucleoCounter® (Chemometec) according to the manufacturer's guidelines. Cells

were disaggregated by the same method explained above and centrifuged at 4000rpm for 5 min. The supernatant was discarded and cells were resuspended in 1mL medium. For cell quantification, 40µL of the resuspended cells were mixed with 40µL of lysis buffer or solution A (Chemometec) and 40µL of stabilization buffer or solution B (Chemometec). Nuclei were then counted using a NucleoCassete™ (Chemometec) on a NucleoCounter ® machine (Chemometec).

2.6.5. Cell treatment

For treatment, cells were seeded in 6-well plates (Corning). Cells from T75 cells were disaggregated using TyrpLE as explained above. After disaggregation, cells were resuspended in 1 mL medium for being counted with the assistance of a nuclecounter as explained in the method above. Cells were then diluted to a final concentration of 200,000 cells in each well in a final volume of 5mL. The medium was supplemented with the different drugs and treatments depending on each experiment. A first set of experiments included paracetamol, ibuprofen and a combination of prostaglandin EP2+EP4 antagonists to see if consistent treatment effects were obtained and to study the possibility that the effects seen are a consequence of disruption of the prostaglandin E2 pathway. For these reasons, cells were treated with 10 or 50µM paracetamol, 10µM ibuprofen or 10µM L-161,982 (EP2 antagonist) + 10µM PF04418948 (EP4 antagonist) for mechanistic experiments (Figure 2.8)

After establishing the effects of paracetamol on NT2 cell number, it was decided to perform a rescue experiment, using EP2+EP4 agonists alone or in combination with paracetamol. NT2 cells (excluding vehicle and 10 or 50µM paracetamol alone) were first exposed to 10µM Butaprost (EP2 agonist; Abcam) and 10nM CAY10598 (EP4 agonist; Abcam) for 4h before the medium was changed to the subsequent treatment: 10µM Butaprost + 10nM CAY10598, 10 or 50µM acetaminophen or the combination of acetaminophen + EP2/EP4 agonists (Figure 2.8). The total length of

the exposure was 48h. This experiment was performed in 6-well plates, following the same method as explained above.

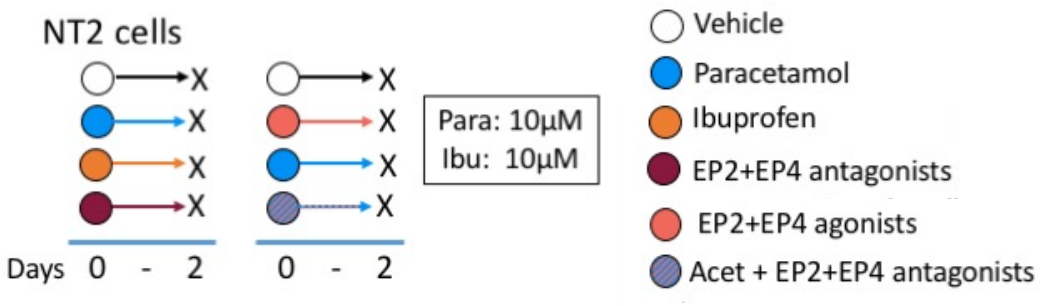


Figure 2.8. Representation of NT2 model exposure regimes. Cells were exposed to different treatments for 48h days.

For the mechanistic analysis, experiments were repeated 3 times (n=3), on different days, each time in triplicate with two different treatments per 6-well plate. Each set of replicates were performed in duplicate: one for flow cytometry and one for RNA/protein extraction. The rest of the experiments were performed 3 times (n=3), but only one set of triplicates was made.

In light of some of the results in chapters 3 and 5, which found treatment-induced modification of the expression of epigenetic regulatory genes, it was decided to investigate if these effects had consequences at the protein level. To do so, Elisas for H3K27me3 and DNA methylation (5-mC and 5-hmC) were used. NT2 cells (n=3 experiments, each in triplicate), treated with 48h exposure to vehicle, 10µM paracetamol, 10µM ibuprofen or 10µM EP2 antagonist + 10µM EP4 antagonist were collected by TRyplE as outlined earlier. For these experiments, treatment was done in T75 flasks to obtain a higher number of cells for the experiments. Two million cells were seeded in each T75 and cultured with the appropriate treatment for 48h. Each experiment was performed on a different day, in duplicate, one for the study of H3K27me3 and the other for the study of DNA methylation. For cell disaggregation, 3mL TRypleE was used, neutralized with 7mL PBS. Cells were then transferred into

15mL falcon tubes and centrifuged at 2,000 x g for 5 mins. After the supernatant was removed, cells were resuspended in 1mL PBS and transferred into Eppendorf tubes for a further wash with PBS. After the last wash, the supernatant was removed and cells were stored at -80ºC until all the culture experiments were performed.

2.6.6. RNA/protein extraction

RNA and proteins were extracted from one set of experiments using TRIzol (Zymo Research). Cells were first washed twice with pre-warmed PBS. NT2 cells were then treated with 1mL TRIzol, which causes cell disaggregation and disruption. The solution was then transferred into a 2mL Eppendorf and instantly frozen at -80ºC until all the experiments from the same set were finished. TRIzol reagent was used because it allows a phase separation and enables RNA and protein to be isolated from the same samples, so final results can be compared. For extraction, all of the samples were thawed on ice before adding 0.2 mL chloroform to each tube. After a 3 min incubation, samples were centrifuged for 15 mins at 12,000g at 4ºC. The centrifugation separated the samples into 3 different phases: a lower red phenol-chloroform phase containing the proteins, an interphase containing the DNA and a colourless upper aqueous phase containing the RNA. The phases containing proteins and RNA were then transferred to different Eppendorf tubes and frozen at -80ºC respectively until further analysis.

2.6.7. Cell cycle analysis

Following previous studies of prostaglandins being able to control cell proliferation, as well as the decreased GC proliferation seen in 1st trimester fetal human gonad cultures, I decided to study the possible effects of the different treatments on cell cycle gene expression in NT2 cells. This was done by flow cytometry after Hoechst staining.

2.6.7.1. Hoechst staining

Hoechst nucleic acid stain is a cell-permeant nuclear counterstain able to emit blue fluorescence only when is bound to DNA. This ability, in combination with flow cytometry, is used for measuring the amount of DNA present in each cell and to then relate that to the different cycle stage of the cell at that specific moment. The cell cycle can be divided into different stages: G_0, G1, Synthesis, G2 and mitosis. The amount of DNA in the different stages will be different, because DNA is only replicated during the synthesis phase. Taking into account the amount of DNA, enables easy distinction between three different phases: G_0/G_1, with the minimum amount of DNA; synthesis, where the amount of DNA is higher than in G_0/G_1 but inferior to the G_2/mitosis, where the DNA is double than in G_0/G_1. Having that in mind, the NT2 cells exposed to different treatments were sorted using different gate settings that were set depending on the amount of signal present (ie. DNA).

NT2 cells were seeded in 6-well plates and exposed to the treatments as previously explained. After the culture, cells were disaggregated with 1mL TRyplE for 5 mins and neutralised with 1 mL prewarmed PBS before being transferred to 1.5mL Eppendorf tubes. Samples were centrifuged at 2000 x g for 5 mins and washed twice with PBS with centrifugations between washes. After the last wash, the supernatant was discarded and cells were resuspended in 200µL solution containing 15µg/µl Hoechst (Cell signalling) in 2%FCS. Samples were then incubated for 30 mins at 37ºC to allow the Hoechst to enter the cells and stain the DNA. After the incubation, the reaction was stopped by adding 1mL PBS and the tubes were centrifuged under the same conditions as before. Two further washes were done with PBS under the same conditions. After the last centrifugation, the supernatant was discarded and the cells were resuspended in 300µL 1% FCS on PBS made up from a 10% FCS frozen Stock. Cells were immediately transferred into ice and stored in the dark until flow cytometry was performed.

2.6.7.2. Flow cytometry

For flow cytometry, a 5 laser LSR Fortessa (BD bioscience, Oxford, UK) flow cytometer was used. This technique measures single cell width, height and amplitude. The flow cytometer exposes the cells to a laser, the light of which is reflected by the cells and this is detected by the machine. In this case, the flow cytometer will also detect the signal produced by Hoechst dye when it is excited and will be able to plot the cells according to the strength of this signal. Hoechst was detected with the UV-450nm laser was used. PI was detected with the 488nm laser.

Cells were also treated with propidum iodide (75µM; BD biosciences) to identify and sort out dead cells. Debris, duplets and dead cells were all removed from the analysis by the software. The resulting cells were then plotted in a single dimension generating a histogram. This plot was then divided into three different regions, also known as gates, based on the fluorescence intensity. Each gate was defined as G_0/G1, Synthesis or G2/Mitosis. For the purposes of this experiment, 10.000 cells were taken into account per sample. An example of a histogram is shown in Figure 2.9.

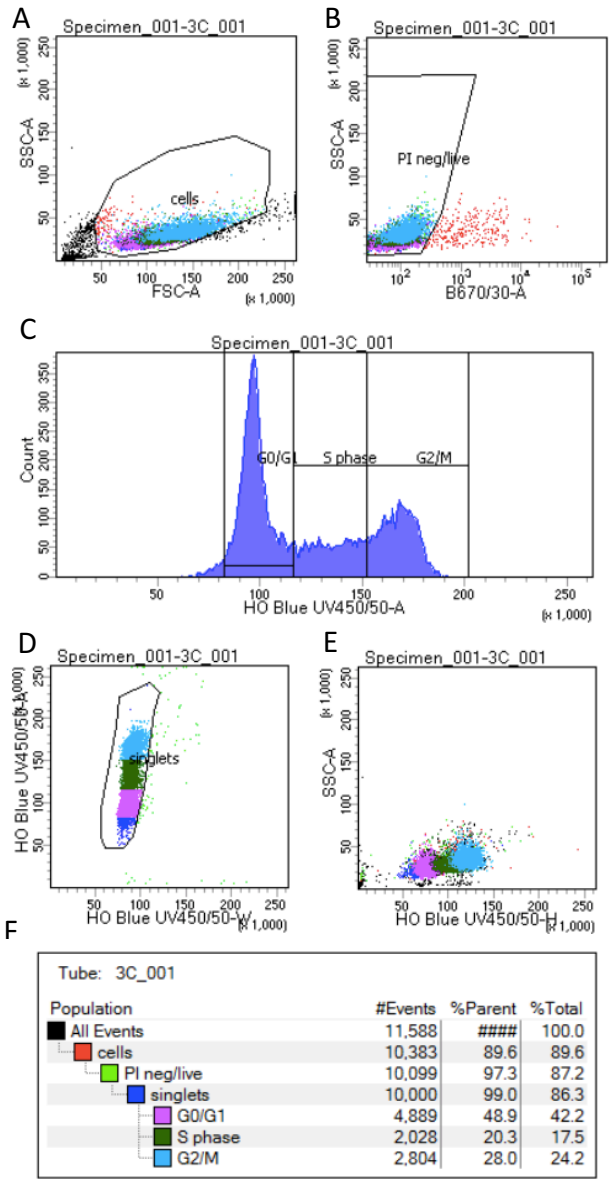


Figure 2.9. Flow cytometry histogram obtained from the study of cell cycle in NT2 cells. **A** Cells were selected based upon size and separated from debris. All cell inside the circle were considered as cells. **B** Dead/alive cells were separated using PI as a marker for dead cells. Cells inside the lines were considered alive and cells outside (red) as dead. **C** Cell count for cells with different DNA content, separated in different gates depending on their cell cycle stage. **D** Single cells were selected and separated from duplets. Cells inside the lines were considered singlets. **E** Cells were separated for DNA content depending on the Hoechst signal they were able to produce (Pink for G0/G1; green for synthesis phase; Blue for G2/Mitosis). **F** Total number of cells separated in each gates including the number of events and the percentage on cells in each gate.

2.6.7.3. Cell death

Cell death was measured by exposure of the cells to Propidium iodide, which is only able to stain dead cells. The flow cytometry software output includes the % of live cells, which was then converted to % of dead cells. This value only takes into account the dead cells that are still present in the cultures and not the those that may have died during the culture process.

2.6.7.4. Analysis

The results obtain by the software, which provide us with the percentage of cells in each gate and the percentage of cell death, were then analysed. Gates were set up before the start of the experiments following previous analyses performed by Maria Camacho. At the end of each experiment, once all the samples were measured (vehicle and treated), the gates were slightly manually corrected, as the intensity of the laser can have small variations.

2.7. Protein detection by Elisa

An H3K27me3 ELISA kit was used to analyze this specific histone modification marker in NT2 cells. Another Elisa kit was used to analyze the presence of PGE2 in the media of the hanging drop human fetal gonad cultures.

2.7.1. Crude extraction

For the H3K27me3 analysis, the crude histones were extracted using 500µL 0.4 M HCL (Sigma) for 30 min, occasionally inverting the tube for a proper homogenization. Samples were then centrifuged at 1000 x g for 10 mins at 4ºC. The supernatant was immediately transferred to a new Eppendorf tube and neutralized with 200µL neutralization buffer containing 1M Sodium phosphate (Sigma), dibasic pH 12.5 supplemented with 2.5mM dithiothreitol (DTT; Sigma) and COmplete ™ protein inhibitor cocktail (Roche), following the manual instructions. The total amount of

protein in these samples was then determined by the Bradford protein assay. Standards (0, 125, 250, 500, 750, 1000, 1500 and 2000ng) were prepared using 2mg/mL stock of albumin (Thermo Fisher) by serial dilution. 5µL of each standard and sample were added to 250µL of Bradford reagent at room temperature (Thermo Fisher) in duplicate, in 96-well plates. Samples were incubated for 15 mins before being read with the spectrophotometer (LabSystems; 595nm wavelength). The standards were used to produce a standard curve to calculate the final concentration of each sample.

2.7.2. H3K27me3 calculation

For the H3K27me3 protein calculation, standards (0, 15.6, 31.25, 62.5, 125, 250, 500 and 1000ng) were prepared using the pure stock of 1µg/µL H3K27me3 provided in the kit, by serial dilution directly on the 96-well plate provided. ELISA plates were already pre-coated with an H3 antibody. All the reagents needed for the protocol, such as the wash buffer and antibody dilution was performed before the start of the experiment following the manufacturer´s guidelines. 50µL H3K27me3 standards or the extracted crude histones from each sample were added to the appropriate well. After incubation, the diluted primary antibody was added and incubated for 1h. A secondary HRP conjugated antibody was then added followed by incubation for 1h. For the colorimetric reaction to start, the developing solution was added, which reacts with the HRP conjugate and produces a colorimetric signal. The stop solution was added after 5 min to stop the reaction and avoid overreaction. All samples were determined in duplicate. Absorbance was measured using a spectrophotometer at 450nm. The signal measured for the H3K27me3 standards was used to produce a standard curve, which was used to calculate total H3K27me3 per well. The relative amount of H3K27me3 in each sample was calculated by dividing by the total protein concentration in each sample, which had been determined earlier.

2.7.3. Prostaglandin E_2 content in culture media

Elisa was also used to calculate the PGE_2 content of hanging drop cultures of 1st trimester human fetal testis samples to investigate if paracetamol or ibuprofen had any effect on it. The medium was collected each day, pooing the medium from all culture pieces from the same fetus/treatment together. This allowed me to get enough medium to perform the ELISA. An ELISAkit (Enzo life science) was used, which uses a monoclonal PGE_2 antibody. Standards were diluted using hanging drop medium to the final concentration specified in the manufacturer´s protocol (2500, 1250, 625, 313, 156, 78.1, 36.9 pg/mL. Positive and negative controls were also used. Each reaction was done in duplicate. The samples and standards were diluted 1:2 in fresh medium to have enough material to do the analysis in duplicate. This dilution was taken into account in the final calculations. The samples were incubated in the wells with the antibody during 2h shaking. The wells were then washed three times with the wash buffer provided in the kit, before adding 200µL pNpp substrate solution and incubated for 45 mins without shaking. The reaction was then stopped by adding 50µL Stop solution (included in the kit). After stopping, the absorbance was measured in each well at 405nm with a spectrophotometer. The intensity of the absorbance (ie. the color produced) is inversely proportional to the concentration of PGE_2 in standards and samples, which thus enabled calculation of the PGE_2 content for each sample.

2.8. RNA analysis

The modification of gene expression as a consequence of the exposure of NT2 cells or rat fetal gonads/cultures to the different treatments was investigated by determining the mRNA expression level of specific genes. To do so, mRNA was extracted and converted into cDNA, which enabled qRT-PCR for the genes of interest to be performed. RNA analysis was performed in rats (in vivo and in vitro experiments) and NT2 cells.

2.8.1. RNA isolation

RNA extraction from the different samples was performed using the PureLink® RNA Micro Kit (ThermoFisher) according to the manufacturer's instructions. All samples were stored at -80ºC prior to the RNA isolation. For NT2 cells, the RNA was firstly extracted using TRIzol reagent (as specified above). For rat studies, RNA was obtained from the tissue using mechanical disruption. In order to yield a sufficient quantity of RNA material for analysis, all tissue pieces from the same gonad were pooled in hanging drop cultures. For in-vivo rat studies, both ovaries from the same fetus were pooled, whilst a single testis from each fetus was ample. For mechanical tissue disruption, samples were placed into a 2mL Eppendorf containing a 5mm diameter stainless steel bead for homogenisation. 350µL lysis buffer, made from RLT buffer (ThermoFisher) and 0.1% β-mercaptoethanol (Sigma), were added to each tube prior to homogenization with a tissue lysser (Qiagen) at 25Hz for 5 mins (2.5 mins in opposite positions), causing tissue disruption and release of RNA. Tubes were immediately centrifuged for 3 mins at 14,000 x g, which helps to remove the fat and other parts of the tissue that could be detrimental for the RNA extraction. The supernatant was then transferred to another 1.5 mL Eppendorf containing 70% ethanol made up from absolute ethanol and RNAse free water (Ambion). The samples were then manually mixed by pipetting and transferred to PureLink mini columns (supplied in the kit) and placed inside a collection tube. Columns were then centrifuged for 15 secs at 14,000 x g. The collections tubes were emptied before adding 350µL wash buffer I to the columns which were re-centrifuged as before. To avoid DNA contamination, DNase on-column digestion was performed by 15 mins incubation with 20µL DNase provided in the kit (10µL DNase + 10 µL Buffer). DNA digestion was stopped by adding 350µL wash buffer I and centrifuging the columns for 15 secs at 14,000 x g. The membranes were then washed twice by adding 500µL wash buffer II followed by a 15 secs centrifugation at 14,000 x g. After emptying the collection tubes, a further 2 mins centrifugation at 14,000 x g was performed to dry the membrane before RNA elution. The columns were then transfer into sterile 1.5

mL tubes and 20µL RNAse free water was added directly over the membrane. After 1 min incubation, the columns were centrifuged at 14,000 x g for 1 min for final elution. RNA samples were kept on ice for quantification or frozen at 80ºC for storage.

2.8.2. RNA quantification.

The concentration of each RNA sample was then quantified at the same time that the quality of the RNA was measured. This analysis was performed in a Nanodrop-1000 spectrophotometer, which measures the concentration in µg/µL and measures the absorbance at 230 260 and 280nm. The ratio between these different absorbances is used to measure the purity of the RNA extracted. The $A_{260/280}$ ratio is commonly used to measure protein and phenol contamination. A ratio close to 2.0 is commonly accepted as being pure RNA. The $A_{260/230}$ ratios is a secondary measure of nucleic acid purity and it is usually accepted to be between 2 and 2.2 for pure extractions. A low $_{A260/230}$ ratio is normally related to residual phenols or guanidine, usually used during the extractions. Once the RNA was measured, the samples were diluted to a final concentration of 100 µg/µL before long term storage at -80ºC.

2.8.3. Reverse transcription

Extracted RNA was converted into cDNA via reverse transcriptase polymerase chain reaction (RT-PCR) using a Vilo kit (Invitrogen) according to the manufacturer's instructions (Table 2.3). A master mix was made by mixing the reagents and 19µL were placed in different 0.2mL sterile thin walled PCR tubes. RNA samples, at a concentration of 100 µg/µL, were added to each tube (100ng). Control tubes were also made, one without RNA and another one without enzyme. Tubes were placed in a thermocycler for cycling on the Vilo program (table 2.3). cDNA was stored at -20ºC for future experiments.

Table 2.3. Regents and program used for a qPCR reaction using Vilo kit

Reaction	Volume per tube (20 µL)
RNase free water	14.75µL
5x Vilo Reaction Mix	4µL
10x Superscript Enzyme Mix	0.25µL
RNA (100 µg/µL)	1µL
Temperature (ºC)	Time (minutes)
25	10
42	60
85	5
4	unlimited

2.8.4. Primer and probe design

RT-qPRC was performed using Taqman technology. Primers were designed using the Universal Probe Library assay design centre, provided by Roche. This system allows the design of specific primers for the gene of interest and at the same time identifies the probe to use. Designed primers need to be complementary to the 3' end of the sense and antisense strand of a specific region of the target cDNA. These primers are species specific, so, when required, a different set of primers was designed for human and rat genes. To avoid possible DNA amplification, primers were designed so that the probe spans introns. The system provides different primer/probe combinations and one was selected according to the availability of probes. Forward and reverse primers were (Eurofins) reconstituted in RNAse free water to a final concentration of 100µM, according to the manufacturer´s conditions, for long term storage (-20ºC). For RT-qPCR, primers were then diluted to a concentration of 20µM. The primer sequences and the probe number used for each gene of interest are listed in Table 2.4.

Table 2.4 Primers for qPCR analysis.

Rat primers				
Name	Forward (5'-3')	Reverse (5'-3')	Probe	Cat. no.
Aldh1a1	caagctggctgacttaatgga	ccaccattgatggcctct	#84	04689089001
Aldh1a2	ggacgcttctgaaagaggac	ccgccatttagtgattccat	#120	04693540001
Amh	ctggacaccgtgcctttc	cactgtgtggcaggtcctc	#26	04687574001
β-catenin	gcgctggtgaaaatgctt	ctaggcgcactgccattt	#12	04685113001
Bdnf	agcgcgaatgtgttagtggt	gcaattgtttgcctcttttdt	#92	04692098001
Cyp26b1	gagagaggagagaggctggata	ggctgcactggctgtagttt	#49	04688104001
Dact1	cctggaggagaacatcttgc	caacaaaccggcatctcttc	#16	04686896001
Dact2	cctgcgaggccttagaca	cggcatcactcagctcatag	#40	04687990001
Dact3	agcagctgggagacctga	cggtagaactggggtcttca	#109	04692284001
Dmrt1	cagaagccaaagcaagtgtg	agctgctggagagggaaac	#129	04693655001
Dnmt3a	aacggaagcgggatgagt	gcaatcaccttggctttctt	#75	04688988001
Dnmt3b	caaatccagggacttgcag	acccactagcaccctcttctt	#94	04692110001
Egfr	acagcgctaccttgttatccaa	cagcatcaactacgtcttccat	#63	04688627001
Ereg	aggcgccttgctcctatc	actgcttgtagaaggtgggaac	#58	04688554001
Ezh2	gactggtgaagagttgttcttga	ctcgttcgatgcccacata	#122	04693566001

Foxo3	ttcaaggataagggcgacag	ggctgtgcagtgacaggtt	# 58	04688554001
Inhhba	cggagatcatcacctttgc	tcactgccttccttggaaat	# 98	04692152001
Jmjd3	ttcgtccttccaggagtcac	ggttcctcggattcctcatc	# 63	04688627001
LDS1	gagacggccaagcatcag	ccactgatgatgtctgggaa	# 38	04687965001
Notch2	tgcctgtttgacaactttgaa	agtggtctgcacagtatttgtca	# 6	04685032001
Oct4	gaagttggagaaggtggaacc	ccttctgcagggctttcata	# 95	04692128001
Rarα	ttggaatggctcaaaccac	agggctgggcactatctctt	# 15	04693493001
Rarβ	agccaccggcatactgctc	cagacgaagcagggcttg	# 70	04688937001
Rarγ	aagccaccaggagactttc	cgctggagttcgtggtgta	# 53	04688503001
Rxrα	gctccatagctgtgaaagacg	agctccgttagcaccctgt	# 79	04689020001
Rxrβ	gttcttccatggggtctcct	ggagcgacactgtggagttaat	# 66	04688651001
Rxrγ	gggcatgaagagggaagc	ctggcacattctgcctcac	# 13	04685121001
Rspo-1	cgacatgaacaaatgcatca	ctcctgacacttggtgcaga	# 5	04685024001
Sox9	atcttcaaggcgctgcaa	cggtggaccctgagattg	# 63	04688627001
Sp1	gcacaggcagtagcagca	tgctgttctcattgggtgac	# 84	04689089001
Sp2	tcaacatcaaggtgtccaag	gcactgtcagctgttgctgt	# 9	04685075001
Sstr1	ttttgcacagccttactgtca	ttgagagtagaaaagaccagttgc	# 1	04684974001

Tet1	gagggaaaagaagcccaa a	aacaaacccaacctttgctc	# 60	04688589 001
Wnt4	gtcctcgccgtgttctcg	cttctgcacgtttcctctt	# 44	04688040 001
Human primers				
TET1	gatgacagaggttcttgca cat	aggttgcacggtctcagtgt	# 86	04689119 001
EZH2	aagaagagaagaaagatg aaacttcg	ttggtgtttgacaccgagaat	# 122	04689054 001
DNMT3a	cctgaagcctcaagagcag t	tggtctccttctgttctttgc	# 46	04688066 001
DNMT3b	ggtgcactgagctcgaaag	aagaggtgtcggatgacagg	# 3	04685008 001
OCT4	cttcggatttcgtcttctcg	cttagccaggtccgaggat	# 22	04686969 001
AP2γ	gagccaaatcgaaaaatg ga	gccaaatgaacagcttcacc	# 7	04685059 001
NANOG	atgcctcacacggagactg t	cagggctgtcctgaataagc	# 69	04688686 001
TBP	gcccatagtgatctttgcag t	cgctggaactcgtctcacta	# 67	04688660 001
WNT4	cgtcttcgcctcttctc	ggatcaggcccttgagtttc	# 63	04688627 001
β-CATENIN	gcccaggacctcatggat	cacaatgcaagttcagacaa taca	# 16	04686896 001
E2F2	aggggaagtgcatcagag tg	ccagcgaagtgtcataccg	# 7	04685059 001
CCNE1	gggacaccatgaaggagg a	tcttcatctggatcctgcaa	# 1	04684974 001
HIST1H4B	aagtgctgcgggataacat c	ttaaccccaccacgccta	# 9	04685075 001
RRM2	tggacctctccaaggacat t	ggctaaatcgctccacca	# 48	04688082 001

CCNA2	catgtaccctggatcttttattgg	tgcattgcttcagactccac	#7	04685059001
CCNB1	cctccggtgttctgcttc	ttcagcattaattttcgagttcc	#74	04688970001
BUB1	gcaacaaaccatggaactacc	ttctaaaaagaccttcaggctaca	#63	04688627001
RAD21	attgacccagagcctgtgat	ggggaagctctacaggtggt	#62	04688619001
PPTG1	gcctctcatgatccttgacg	gcttgaaggagactgcaaca	#22	04686969001

2.8.5. Quantitative PCR

Quantitative real-time/reverse transcriptase polymerase chain reaction (RT-qPCR) was performed, which is able to amplify the target cDNA sequence at the same time as it quantifies the amount of that cDNA present. RT-qPCR follows the same basic principles as classic PCR with different phases with changes in temperature allowing: 1. *Denaturation* phase, where the double stranded DNA converts into single stranded DNA (ssDNA) at 95ºC by disrupting the hydrogen bonds holding the strands; 2. *Annealing* phase, where the primers and probe link with the ssDNA at 60ºC; 3. *Extension* phase, where the polymerase enzyme binds the primer/template hybrid and starts the synthesis. If there are no limiting factors, each cycle should double the amount of the DNA amplicon, resulting in an exponential amplification. RT-qPCR is normally run for 40 cycles, producing two different phases: a linear phase, where the amplification is exponential, and the plateau phase, where the amplification has reached a maximum amount. This technique enables identification of the number of cycles of PRC needed to reach the plateau and hence to calculate the relative amount of cDNA present in the sample. This value is an indirect measure of the total amount of mRNA present and therefore, the level of expression of the gene of interest.

2.8.6. Taqman RT-qPCR

RT-qPCR was performed using Taqman technology, which uses a fluorescent reporter molecule (probe) able to detect the PCR product as it is produced by the consecutive

cycles of PCR. The more PCR product, the more fluorescence emitted. These probes are formed by a DNA sequence (able to link to the gene of interest) which contains at the 3´end a non-fluorescent quencher (6-carboxy-tetramethyl-rhodamine: TAMRA) and a reporter dye (6-carboxyfluorexcein: FAM) at the 5' end. The former maintains the probe in a non-fluorescent state. During the denaturation step (producing single stranded DNA) of the different PCR cycles, if the target cDNA is present in the sample, the probe attaches to its target region at the same time as the forward and reverse primers. Taq polymerase enzymes are able to bind to the 5' end of the primer and start the polymerase reaction. Once it reaches the probe the FAM dye is cleaved and released, emitting a readable signal by the qPRC machine, thus allowing the amount of fluorescence present to be recorded. With each PRC cycle, more probe will be linked to cDNA and more reporter is cleaved producing more fluorescent signal. This method allows quantitative measurement of the level of fluorescence produced (ie. The amount of PCR product). (Figure 2.10)

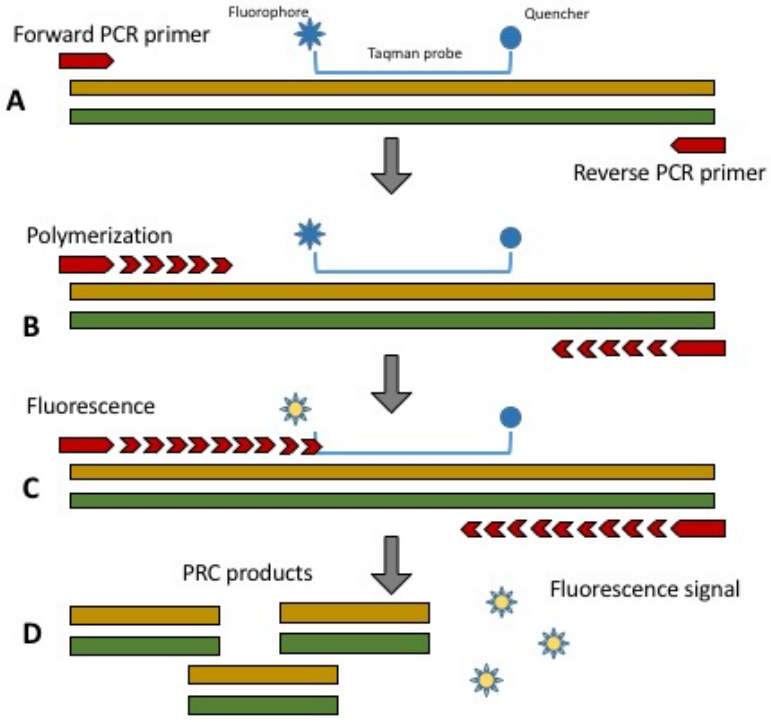


Figure 2.10. Principles of Taqman qPCR. **A** Taqman probe and PRC primers link in specific locations of the double stranded DNA. **B** Elongation phase. **C** Taqman

polymerase removes the fluorophore from the Taqman probe as a result of the elongation. **D** PRC product is finished and fluorophore produces a fluorescence signal, which is detected by the machine.

RT-qPCR reactions (in duplicate) were performed in 96 or 384-well MicroAmp optical reaction plates (Applied Biosystems). The level of expression of the studied genes was related to an internal reference gene, a house keeping gene, used as a reference for RNA variation between samples. In the case of rat studies, only 18s, a ribosomal RNA, was used because of the low amount of RNA obtained from the samples. In the case of NT2 cells, an extra reference gene was added: TATA-box binding protein (TBP). These genes were chosen from a list of recommended candidates of housekeeping genes for gonad studies (O'Shaughnessy et al., 2011). The expression of TBP and beta-2-microgobulin (B2M) were studied with the different analgesics treatment, TBP being the one showing a lower variation among samples (Figure 2.11). 18s was analysed in the same well as the gene of interest (rat studies) or the same well as TBP (NT2 studies) using an 18s Taqman Ribosomal RNA control reagent, which uses a VIC fluorescence report. 18s master mix was made by diluting 53µL of 18S ribosomal probe with 53µL of each primer in 150µL TE buffer (Invitrogen). Negative controls (two produced in the cDNA conversion and one extra made with no RNA) were included in all runs. All Taqman reagents, primers and samples were vortexed and kept on ice during the preparation of the plate. Primers were also centrifuged every time before their use to avoid cross contamination. A mastermix was made following the manufacturer´s protocol (Table 2.5) and a volume of 13.5µL, along with 1.5µL cDNA was used in each reaction. An optical adhesive cover (Applied Biosystems) was used to cover the plate. All plates were centrifuged for 15 seconds using a plate centrifuge (Fisher Scientific) before being run on an ABI PRISM 7900 sequence detection system (Applied Biosystems), using the program suggested by the manufacturer (Table 2.5).

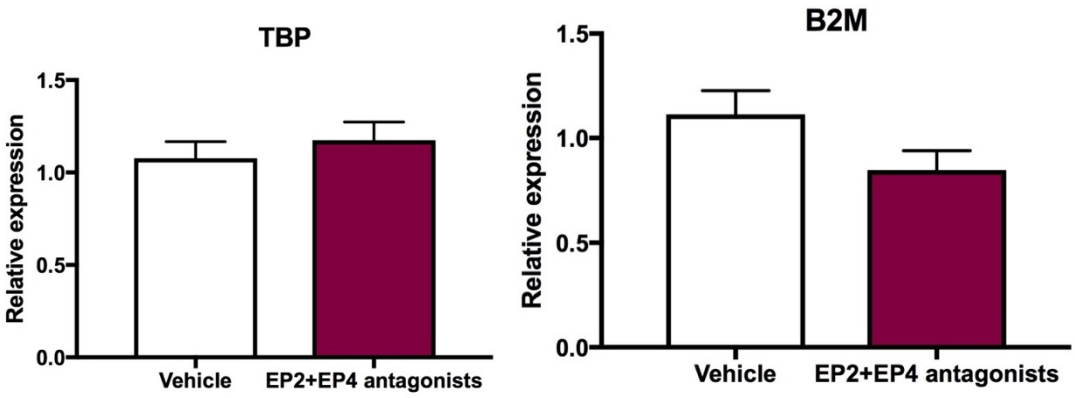


Figure 2.11. Relative expression of housekeeping genes in NT2 cells. TBP (Left) and B2M (Right) after vehicle or EP2+EP4 antagonists exposure were studied as potential housekeeping genes for the qPCR studies on NT2 cells.

Table 2.5. Reagents and program used for Taqman RT-qPCR

Reaction	**Volume per well (15µL)**	
RNAse free water	5.325µL	
Taqman Master Mix	7.5µL	
18s mix	0.225µL	
Universal probe	0.15µL	
20µM forward primer (5' – 3') 20µM	0.15µL	
20µM reverse primer (3' – 5')	0.15µL	
cDNA	1,5µL	
Temperature (ºC)	**Time (seconds)**	
95	180	
95	5	x 40 cycles
60	15	

2.8.7. Primer validation

Because primers are designed following theoretical rules, it does not take into account all the different interactions that they can suffer in normal conditions and can sometimes result in primers not working or not amplifying the target cDNA correctly. In order to test the validity of the designed primers, each pair was

independently studied. To do so, a serial dilution of cDNA (1, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) was used, with the same concentration of primers, to run RT-qPCR and obtain a value of amplification (Ct value, explained below) for each cDNA dilution. If the set of primers was able to produce the correct amplification of the region of interest from the cDNA, there should be an exponential correlation between the cDNA concentration and the number of cycles resulting from the RT-qPCR. This was calculated by plotting the Ct value of each sample against the log transformed relative concentrations of cDNA used. The slope of the curve obtained was used to determine the efficiency of the PCR. This efficiency was calculated as E=10 -1/slope. This result was then transformed to a percentage. A valid efficiency was described as one between 90 and 110%, as it means that during each PCR cycle there is a doubling of the amount of amplicon present in the tube. If primers failed the test, another set of primers from the list produced by the Universal Probe Library assay, was selected and tested.

2.8.8. Analysis of results

RT-qPCR results are represented on an amplification plot given by the ABI software (Thermofisher). In this plot, the amount of fluorescence produced in each well is plotted against the number of cycles. Indirectly, it shows the amplification or the region of cDNA of interest. In the plot, there are 4 distinguished points of interest: the baseline, which refers to the basal fluorescence in the reaction plate (a), the background fluorescence (b), the exponential growth phase (c) and the plateau (d). The exponential phase starts once the level of fluorescence has increased enough and this is referred to as the threshold. Hence, the threshold is manually set at the beginning of the exponential phase. The signal detected above this threshold is defined as the cycle threshold (Ct value) and is the value that is used for statistical analysis.

2.8.9. Relative quantification: ΔΔCt method

I used the ΔΔCt method that allowed me to compare variations in gene expression between the vehicle-exposed and treated samples (Livak & Schmittgen 2001). This method works under the assumption that the amplification efficiency of the genes of interest and the housekeeping genes are similar. This method uses the formula: $X_n=X_0 \times (1+E_x)^n$, where X_n is the number of target molecules at threshold level, n= cycle number, X_0=number of target molecules at the start, $1+E_x$= the efficiency of the target amplification. This formula can be used to derive $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen 2001). ΔCt is calculated for each sample as the Ct value of the reference gene subtracted from the Ct value of the gene of interest. When two different reference genes were used, the average of both Ct values was subtracted from the Ct value of the gene of interest. Each Ct value is obtained from the average of the two Ct values obtained, as each reaction was performed in duplicate. ΔCt was then averaged and compared with a reference (the average of the vehicle samples from that run) in order to obtain the ΔΔCt. This value was used in the formula previously explained ($2^{-\Delta\Delta Ct}$) to obtain a final measure of relative quantification, which was used to compare between vehicle and treated samples. In order to create a graph where the patterns and trends in gene expression are easier to understand, the final values obtained were then normalised to the respective vehicle. This was done by dividing the final value between the average of all the values of the vehicles. All calculations were performed using Excel 15.0 before analysing the statistical significance in GraphPad Prism 7.0.

2.9. Statistical analyses:

Statistical analyses were performed with different systems depending on the models. As each sample came from a different individual (which are considered as being independent) in the rat in vivo and in vitro studies, analysis was performed using one way ANOVA prior to 2-tailed t tests where appropiate. A priori, gene expression data that exhibited a skewed distribution were to be log- transformed before t tests were

conducted to satisfy the prerequisite assumptions of normality. However, none of the results shown in this thesis needed this transformation. For human tissue studies, including fetal gonad cultures and xenografts, results were analysed by two-way ANOVA. This took into account for inter-individual variation between fetuses and inter- replicate differences from the same fetus (as the size of these varied and could not be standardised), as previously described (Mitchell et al., 2012). Two-way ANOVA was also used for the NT2 cell studies. This took into account for the variation existing between different experiments (performed on different days; n number) and culture replicates within the same experiment. Although the latter could have been averaged to yield a single mean value, this would have discarded data on experimental error (ie the variation between replicates), and it was considered that retaining this represented a more robust and searching analysis of the data. Values in the graphs are expressed as means ± SEM, where appropriate. The criteria for significance, indicated by asterisks, was set at P< 0.05=*; P< 0.01=**; P< 0.001=***; P< 0.0001=****. All statistical analyses and graphs were performed using GraphPad Prism 7.0.

2.10. Common solutions

2.10.1. Hanging drop cluture medium

8.4 mL Culture medium (Alpha-MEM; Lonza)

1 mL Fetal bovine serum (Life Technologies)

100µL Penicillin/streptomycin (Sigma-Aldrich)

100µL L-glutamine (Gibco)

100µL MEM NEAA non-essential amino acids (100X) (Gibco)

200µL Sodium pyruvate 100mM (100X) (Gibco)

1 µL 0.1U/ml human chorionic gonadotropin (hCG) (Preg- nyl, Organon Laboratories)

This was enough to make 10mL, which was then aliquotted into different tubes and supplemented with the appropriate treatment for the culture.

2.10.2. Tris-Buffered Saline (TBS)

605g Tris (Sigma)

876g NaCl (Sigma)

Made up in 8L with dH20, adjusted to pH 7.4 using concentrated HCl (VWR chemicals). The solution was then made up to 10L and stored as a 10x stock solution at 4ºC.

2.10.3. 0.1M Citrate Buffer

42.02g citric acid monohydrate (Sigma)

Made up to 1.8L with dh20, adjusted to pH 6.0 using concentrated NaOH (VWR chemical). The solution was then made up to 2L, filtered and stored at 4ºC. For antigen retrieval, it was used at 0.01M.

2.10.4. Chicken immunochemistry blocking buffer

20mL Chicken serum (Diagnostic)

5g Bovine Serum Albumin (Sigma)

This was made into a 10mL solution with TBS and stored in 5mL aliquots at -20ºC.

2.10.5. NT2 culture medium

500 mL Culture medium (DMEM; Life technologies)

50 mL Fetal bovine serum (Life Technologies)

5 mL Penicillin/streptomycin (Sigma-Aldrich)

5 mL L-glutamine (Gibco)

Medium was made up and stored at 4ºC until needed.

2.10.6. FACS buffer

0.2mL Fetal bovine serum (Life Technologies)

9.8 mL PBS

Chapter 3: Effect of analgesic exposure on fetal rat gonads

3.1. Introduction

Chapter 1 introduced the different publications that have studied the effects of analgesics during pregnancy so far. The majority of these are epidemiological studies and several have shown an association between analgesic exposure and male reproductive disorders, such as cryptorchidism and hypospadias (Snijder et al., 2012; Kristensen et al., 2011). As detailed in section 1.2.2, cryptorchidism can be related to reduced fetal testosterone levels, which is one reason why studies have focused on the effects of analgesics on Leydig cell function, such as testosterone production, in vivo and in vitro in rodent models, but also in in vitro human models (Kristensen et al., 2012; Mazaud-Guittot et al., 2013). However, few studies have investigated the actual mechanism by which analgesics induce their gonadal effects. One possible effect of paracetamol and ibuprofen on fetal gonads is through the PG pathway, as both have been shown to be able to modify this pathway. Moreover, fetal somatic and germ cells in the gonads express PGE_2 receptors in rats and humans (Dean et al., 2016; Bayne et al., 2009).

A few recent studies have used rodents as models in order to study in vivo the effects of these analgesics, focusing in more detail on the fetal gonads (Kristensen et al., 2011; Dean et al., 2016)6. Yet, one of the limitations of these studies is the fact that it is difficult to separate the direct effects of the analgesics on the fetal gonads from the indirect effects that could derive from the effects of the analgesics on the mother. To study the direct consequences of common analgesics on the fetal gonads, some studies have also used in vitro fetal gonad cultures (Kristensen et al., 2012; Mazaud-Guittot et al., 2013).

In this chapter, I will describe the different studies that were performed using in vivo and in vitro rat models exposed to analgesics. The in vivo model, using pregnant rats, was used to study the effects of analgesic exposure on the expression of genes with

important roles during gonad development, including those related to retinoic acid regulation, important during gonad sex differentiation, involved in GC development and in epigenetic regulation. Pregnant rats were exposed to paracetamol or indomethacin from e13.5 onwards, an important time point for gonad development. For example, as described in section 1.3, after undifferentiated gonads have decided a pathway to follow (testis or ovary), important reconstruction of the gonad occurs which includes important GC changes. Thus, ovarian GCs are in the process of entering meiosis and they have already started expressing important factors for this process such as *Notch* or *Stra8* (Le Bouffant et al., 2010). On the contrary, meiosis is blocked in male GCs until puberty thanks to an active process that needs to be continuously regulated (Bowles & Koopman, 2010; Guerquin et al., 2010). Furthermore, male GCs are still actively proliferating, but during the stage of development studied here, they are becoming ready to enter mitosis arrest that will stop their division until pre-puberty (Hassold & Hunt, 2001).

The in vitro model, fetal rat gonad cultures, was used to study the effect of paracetamol and ibuprofen on epigenetic regulators. Moreover, to investigate the possible analgesic mechanism of action, these fetal rat gonad cultures were also exposed to PGE_2 receptor (EP2 and EP4) antagonists to study if the phenotypes seen with this treatment mimicked the effects of analgesic exposure.

3.1.1. Rationale for my studies

Several studies in the Sharpe group were undertaken to investigate the effect of paracetamol and indomethacin on rat fetal gonads, and my specific role was to investigate the mechanism of action of these analgesics and find possible detrimental effects on gonad development, with a special focus on the GCs. The samples analysed were obtained from experiments that were being performed by different members of the lab in order to study the effect of paracetamol and ibuprofen/indomethacin on fetal gonads, with a specific emphasis on the masculinization programming

window (MPW) in rodents (Dean et al., 2016). Hence, experiments were done predominantly using e17.5 testes as standard.

In the in vivo rat model, I firstly performed a study focusing on the expression of some important and well-studied testicular and ovarian genes. These genes were selected based on their importance during gonad development or their relationship with male reproductive disorders. This included genes related to retinoic acid, regulators of sexually dimorphic gonad development. Previous studies by this lab on the same rat in vivo model showed that in utero exposure to paracetamol or indomethacin increased expression of Stimulated by Retinoic Acid 8 (*Stra8*) in the fetal ovary on e17.5 and e18.5 (Dean et al., 2016). Stra8, as its name implies, is regulated by retinoic acid (RA) and thus, one focus of my studies was RA, which plays important roles in general fetal development as well as in fetal gonad development. In the rodent gonad RA is important in controlling the start of GC meiosis, as it is required for pre-meiotic DNA replication (Le Bouffant et al., 2010; Baltus et al., 2006). The study of RA was done indirectly, as it is usually performed, with study of the enzymes involved in RA synthesis from Vitamin A (Aldehyde dehydrogenase 1 family ; Aldh1a1, Aldh1a2 and Aldh1a3) and its metabolic inactivation (via Cyp26b1), but also changes in expression of its receptors (Rarα, Rarβ, Rxrα, Rarγ and Rxrγ) (Childs et al., 2011).

As part of the project, I optimized a fetal rat gonad culture system to study first, in order to find the best culture method, based on previous studies done in human and mice (Kristensen et al., 2011; Jorgensen et al., 2015). First, I tried to optimize the size of the cultures. During the hanging drop culture there is no blood supply, and all the nutrients necessary for the cells are taken directly from the supplemented media. Only the surface of the pieces is in direct contact with the media, while the cells in the interior rely on diffusion from the media though the matrix of the gonad piece. Previous studies with human fetal gonad tissue recommended an average size of

$1mm^3$. However, as this is a different model, and because of the difficulty of cutting the small rat gonads into exactly $1mm^3$ pieces, an initial optimization study that evaluated different sizes and culture lengths of the gonads was performed. Thus, fetal ovaries and testes were culture in ½ or in ¼ pieces of the gonad. The ¼ pieces of testes were also cultured for 2, 6 or 10 days. The gonad cultures were exposed to paracetamol or ibuprofen (instead of indomethacin, due to its more common use). Moreover, antagonists of the prostaglandin E_2 receptors, EP2 and EP4, were incorporated to the studies as an extra treatment to study if blockage of the PGE_2 pathway could mimic the results found with paracetamol or ibuprofen. The same gestational age was studied as in the in vivo studies and cultured e15.5 fetal rat testes and ovaries..

3.2. Material and Methods

Pregnant rats were administered paracetamol (350mg/kg/day x1) or indomethacin (0.8 mg/kg/day x1) by oral gavage by William Mungal. In related studies, another NSAID, indomethacin was administered by subcutaneous injection (0.8mg/kg/day x1). Treatments were administered from e13.5 until the day before sampling (either e15.5, e17.5 or e18.5) as explained in section 2.1.3. The doses of paracetamol in rats were calculated from human relevant doses taking the body surface area into consideration (Reagan-Shaw et al., 2008). Following the method in this article, each species has a different conversion factor, depending on the body surface area, which is 37 for an adult human and 6 for an adult rat. Using these conversion factors, 60mg/kg per day in a human is equivalent to 350mg/kg/day in the rat. Furthermore, further analysis by our lab have shown that this dose used in rats is required to achieve similar levels of paracetamol in blood as in humans and to induce measurable effects (van den Driesche et al., 2015). The dose of indomethacin used derives from previous studies in the lab showing that higher doses of this drug produced littler loss and/or maternal gastric bleeding (Dean et al., 2013).

In order to have a more tractable system and eliminate the maternal indirect factor from the direct effects of analgesics on fetal rat gonads, ovaries and testes were cultured in hanging drops for 48h in the presence of paracetamol, ibuprofen or PGE_2 receptor antagonists. For the hanging drop cultures, an optimization of the size of the sample and time of the culture was performed. Samples were immunostained, together with age matched controls (e15.5 and e17.5) for Vasa (GC marker) and Oct4 (Testis only) or Dmrt1 (Ovary only) to study the viability of the cultures and to see if they showed normal development. Fetal rat testis and ovary samples were cultured with paracetamol (10 μM), ibuprofen (10 μM) or EP2+EP4 antagonists [10μM L-161,982 (EP2 antagonist) + 10μM PF04418948 (EP4 antagonist)]. These cultured ovaries and gonads were used to study the effect of the different treatments on gene expression of epigenetic regulators related with the PRC2, such as *Tet1* and *Ezh2* and also the DNA methyltransferases *Dnmt3a* and *Dnmt3b*.

3.3. **Results**

3.3.1. Effect of analgesic exposure in utero on the retinoic acid signalling system

The expression of *Aldh1a1* was increased in e17.5 testes and e17.5-e18.5 ovaries exposed to paracetamol or indomethacin in utero, but this was only statistically significant in testes (Figure 3.1). In contrast, expression of *Aldh1a2* was unaffected in e17.5 testes of analgesic-exposed F1 fetuses (Figure 3.1). On the other hand, the expression of all RA receptors and *Cyp26b1* showed no significant modification in F1 e17.5 fetal testes from analgesic-exposed fetuses (Figure 3.1). Aldh1a1 was also studied at the protein level by fluorescence immunohistochemistry, but no difference of Aldh1a1 expression at protein level was found comparing the vehicle with any of the treatments (Figure 3.2).

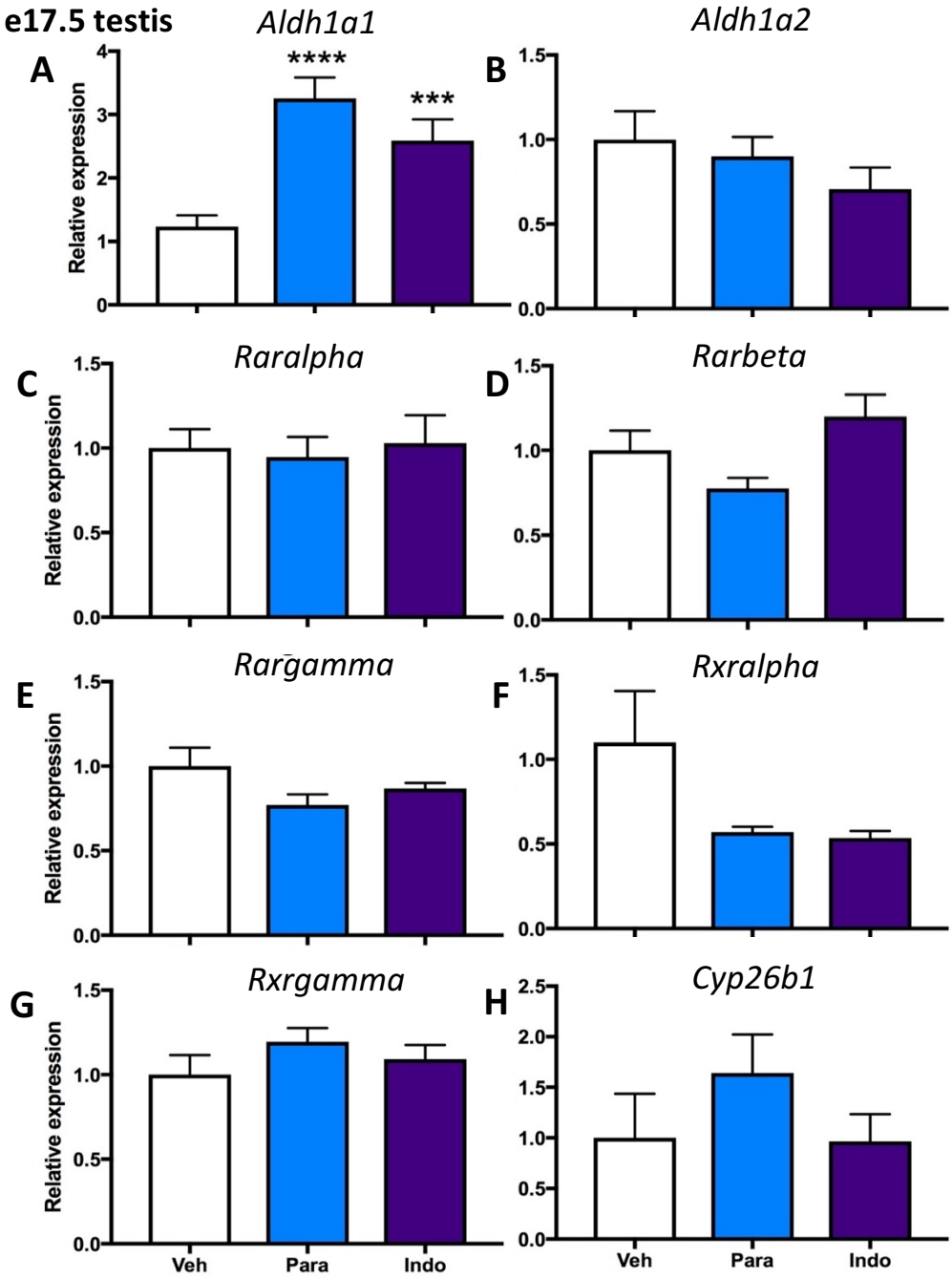


Figure 3.1. Effect of in utero exposure of pregnant rats to paracetamol (Para) or indomethacin (Indo) on mRNA expression of retinoic acid regulators in fetal e17.5 testes. Pregnant rats were administered vehicle (Veh; White), paracetamol (Para; 350mg/kg/day x1; blue) or indomethacin (Indo; 0.8mg/kg/day x1; purple) commencing on e13.5, and fetal gonadal tissue was collected on e17.5, 3 hours after the final maternal treatment. mRNA expression is expressed relative to the vehicle cDNA for the same genes after the treatment exposure. Panels **A** and **B** show the relative expression of the retinoic acid synthesizers *Aldh1a1* and *Aldh1a2*. Panels **C**-**G** show the relative expression of retinoic acid receptors and panel **H** the relative expression of the RA metabolizing enzyme *Cyp26n1*. (Means ± SEM n=11-13). Data were analysed by one way ANOVA prior to a 2-tailed t test ; ***p<0.001, ****p<0.0001, in comparison with corresponding vehicle control group.

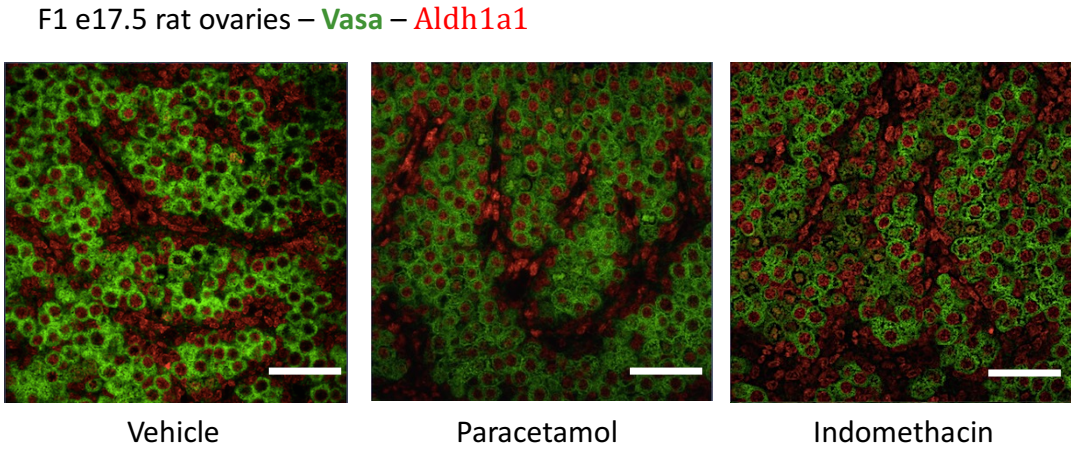


Figure 3.2. Effect of in utero exposure to analgesic on Aldh1a1 in the e17.5 fetal rat ovary. Pregnant rats were administered vehicle, paracetamol (350mg/kg/day x1) or indomethacin (0.8mg/kg/day x1) commencing on e13.5, and fetal gonadal tissue was collected on e17.5, 3 hours after the final maternal treatment. Tissue was immunostained for Vasa (GC - green) and Aldh1a1 (red) Scale bar, 20µm.

3.3.2. Effect of in utero exposure to analgesics on expression of genes involved in fetal rat gonad development

One of the genes found to be affected by paracetamol and indomethacin was Dishevelled Binding Antagonist of Beta Catenin 1 (Dact1), which showed a significantly increased expression in the e17.5 fetal testis (Figure 3.3) and e18.5 fetal ovary (paracetamol only) (Figure 3.4). *Dact 2* and *Dact 3* were also studied, but showed no treatment-induced modification (Figure 3.3). Dact proteins are antagonists of β-catenin, a key player in gonad development, especially in the ovary, but also important in the testis. β-catenin acts together with Wnt protein (see section 1.3.2.1 and Fig. 1.2) and thus, it was decided to focus on the Wnt4/β-catenin pathway. The expression of *Wnt4* in e17.5 testis was reduced by paracetamol exposure only (Figure 3.3). The expression of *β-catenin* was not modified by in utero exposure to either of the analgesics (Figure 3.3) in e17.5 testes. β-catenin is translocated to the nucleus once the pathway is activated and therefore the localization of β-catenin was studied by immunohistochemistry. e17.5 exposed ovaries were studied because of the higher importance of this pathway in ovaries and the availability of analgesic exposed fixed fetal ovaries. However, no change in immunolocalization was found with any of the treatments in e17.5 exposed ovaries, as β-catenin appeared mainly in the cytoplasm of somatic and germ cells (Figure 3.5). The expression of other genes related to the wnt4/β-catenin pathway was also studied, including *Rspo-1, Sox9*, *Amh*, as well as *Gata4*. However, none of these genes showed a significant modification when analysed in e17.5 testes exposed in utero to paracetamol or indomethacin (Figure 3.3).

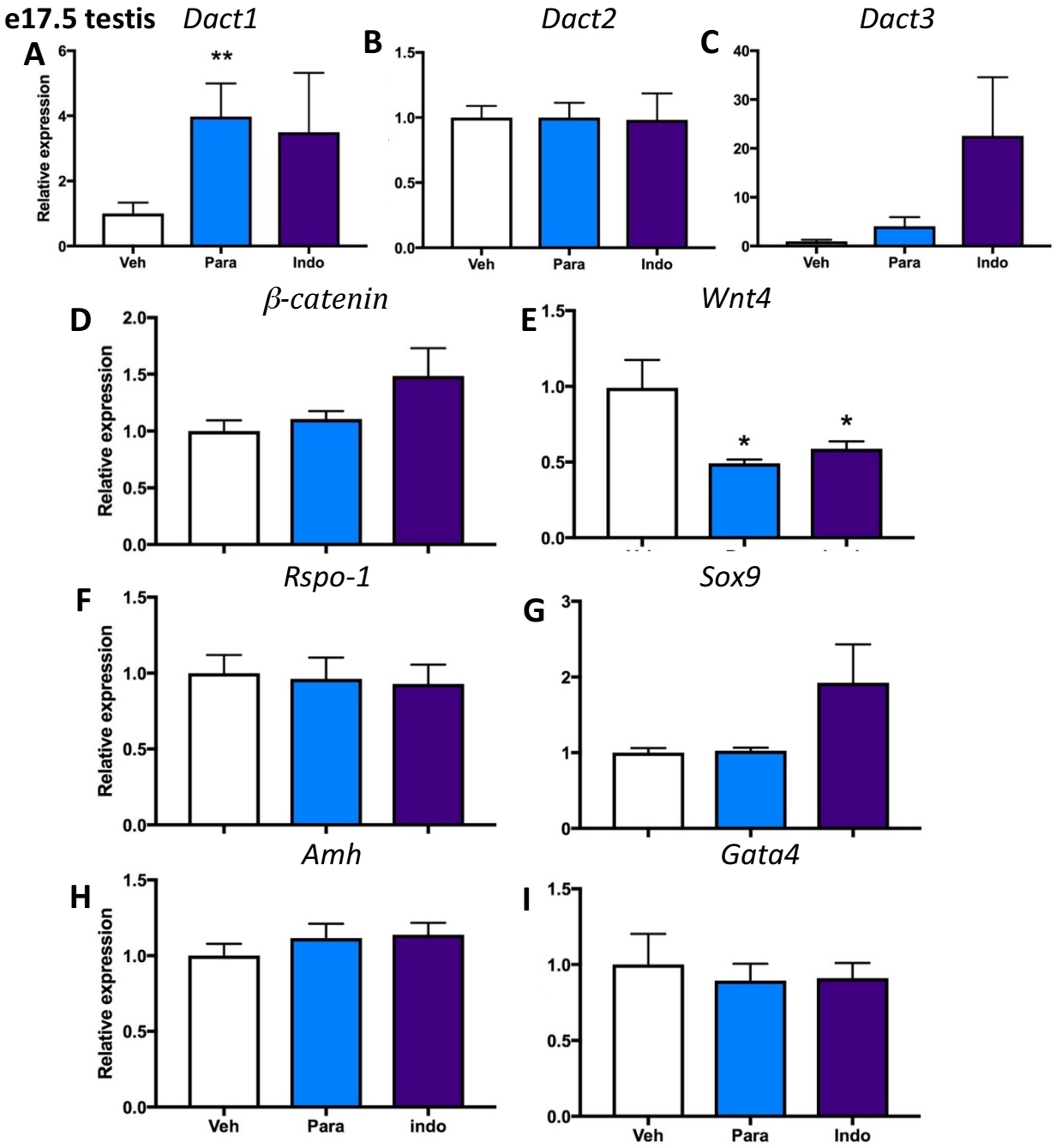


Figure 3.3. Effect of in utero exposure of pregnant rats to paracetamol (Para) or indomethacin (Indo) on mRNA expression of important genes for gonad development in e17.5 testes. Pregnant rats were administered vehicle (Veh; White), paracetamol (Para; 350mg/kg/day x1; blue) or indomethacin (Indo; 0.8mg/kg/day x1; purple) commencing on e13.5, and fetal gonadal tissue was collected on e17.5, 3 hours after the final maternal treatment. mRNA expression is expressed relative to the vehicle cDNA for the same genes after the treatment exposure (Means ± SEM n=11-13). Data were analysed by one way ANOVA prior to a 2-tailed t test; *p<0.05, **p<0.01, in comparison with corresponding vehicle control group.

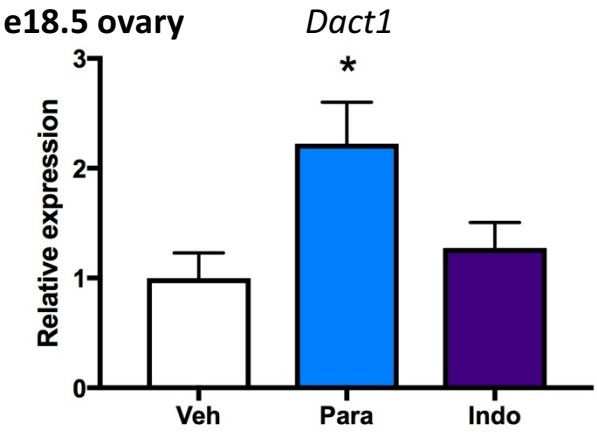
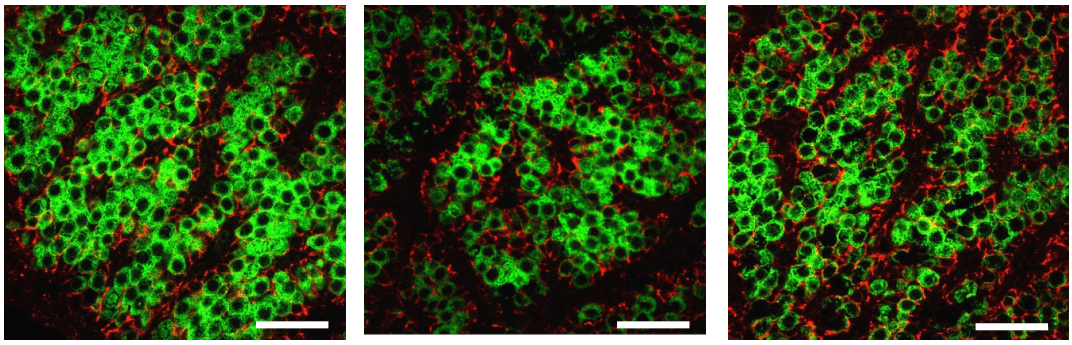


Figure 3.4. Effect of in utero exposure of pregnant rats to paracetamol (Para) or indomethacin (Indo) on mRNA expression of important genes for gonad development in the fetal e18.5 ovary. Pregnant rats were administered vehicle (Veh; White), paracetamol (Para; 350mg/kg/day x1; blue) or indomethacin (Indo; 0.8mg/kg/day x1; purple) commencing on e13.5, and fetal gonadal tissue was collected on e18.5, 3 hours after the final maternal treatment. mRNA expression is expressed relative to the vehicle cDNA for the same genes after the treatment exposure (Means ± SEM n=11-12). Data were analysed by one way ANOVA prior to a 2-tailed t test; *p<0.05, in comparison with corresponding vehicle control group.

F1 e17.5 rat ovaries – **Vasa** – ***β*-catenin**



Vehicle Paracetamol Indomethacin

Figure 3.5. Effect of in utero exposure to analgesic on β-catenin localization in the e17.5 fetal rat ovary. Pregnant rats were administered vehicle (left), paracetamol (350mg/kg/day x1; middle) or indomethacin (0.8mg/kg/day x1; right) commencing on e13.5, and fetal gonadal tissue was collected on e17.5, 3 hours after the final maternal treatment. Tissue was immunostained for Vasa (GC - green) and β-catenin (red) Scale bar, 20µm.

3.3.3. Effect of in utero exposure to analgesics on fetal GC development and follicle pool

Previous rat studies by our lab using in vivo exposure to paracetamol and indomethacin showed male and female GC loss during fetal life. This GC reduction was compensated for postnatally in males, but these effects had long-term consequences in females including a lower adult ovary weight (Dean et al., 2016). The development and proliferation of fetal GC is regulated by a variety of known factors, although the process as a whole is not fully understood. The present studies focussed on the effect of paracetamol and indomethacin on some of the most well-known regulators of GC development and formation of the follicle pool, including Forkhead box O3 (Foxo3), Notch2, Epiregulin, Epidermal growth factor receptor (Egfr) and Inhibin alpha (Inha) (John et al., 2008; Xu & Gridley, 2013; Feng et al., 2014b), as well as Dmrt1, which has been related to GC development, especially in males but also in females (Krentz et al., 2011; Matson et al., 2011). Many of these factors are more relevant to the ovary than the testis. However, due to other ongoing experiments in the lab, the only tissue available for my research was e17.5 testes. These samples were used to study genes important in ovarian development because these genes are also expressed in testis and the mechanisms that regulates them are potentially similar in both sexes.

The expression of *Foxo3*, *Notch2*, *Epiregulin*, *Egfr* and *Notch2* was analysed by RT-qPCR and showed an increase in expression of all these genes in the e17.5 testes of F1 males that had been exposed in utero to paracetamol (Figure 3.6). Paracetamol exposure also decreased the expression of *Inha* (Figure 3.6). Indomethacin exposure did not alter the expression of *FoxO3*, *Notch2* or *epiregulin*, but it increased the expression of *Egfr* and reduced the expression of *Inha* in the same samples (Figure 3.6). Neither of the analgesics modified *Dmrt1* expression in e17.5 fetal testes (Figure 3.6).

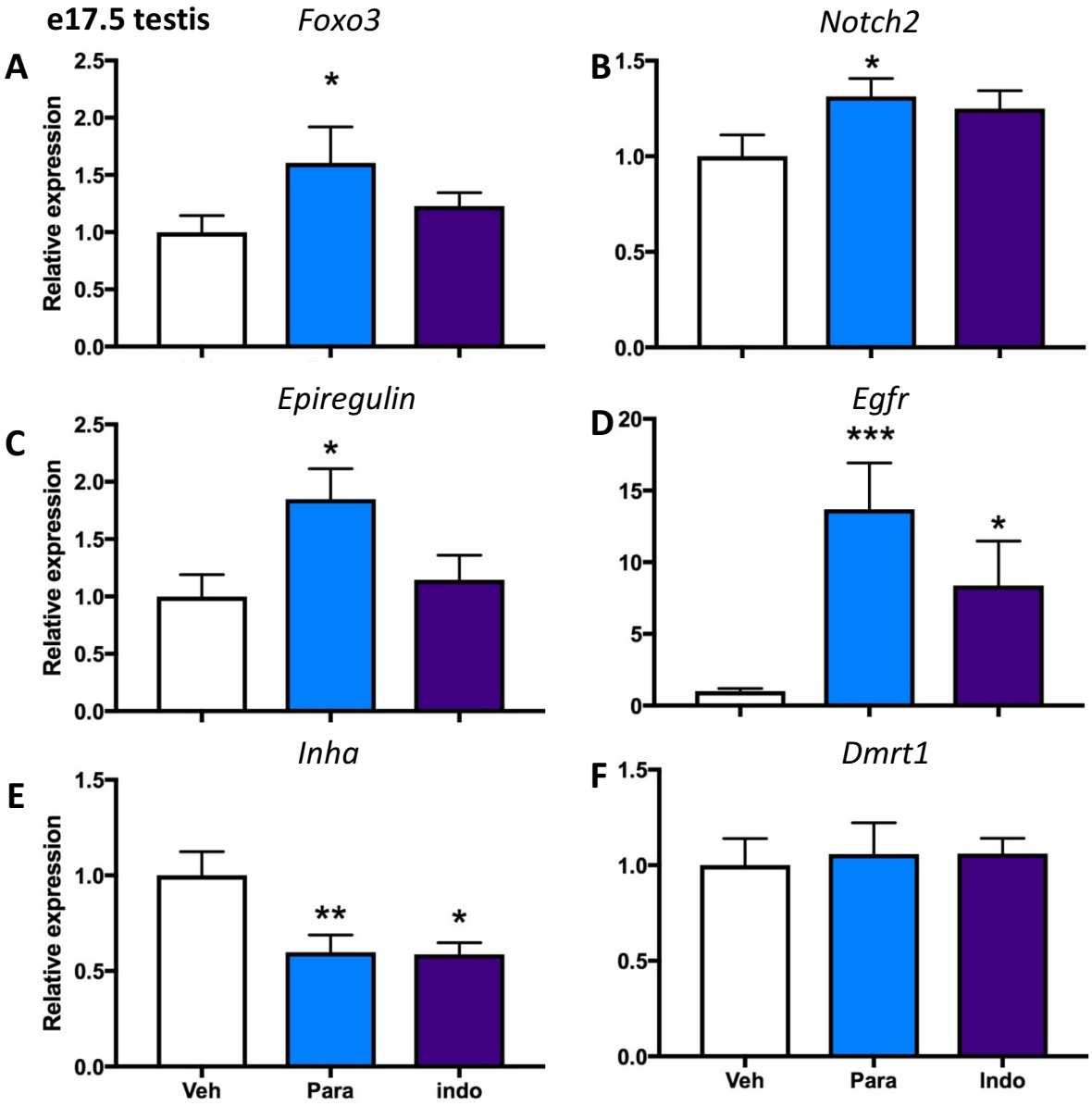


Figure 3.6. Effect of in utero exposure of pregnant rats to paracetamol (Para) or indomethacin (Indo) on mRNA expression of important genes for GC development in fetal e17.5 testes. Pregnant rats were administered vehicle (Veh; White), paracetamol (Para; 350mg/kg/day x1; blue) or indomethacin (Indo; 0.8mg/kg/day x1; purple) commencing on e13.5, and fetal gonadal tissue was collected on e17.5, 3 hours after the final maternal treatment. mRNA expression is expressed relative to the vehicle cDNA for the same genes after the treatment exposure. (Means ± SEM n=11-13). Data were analysed by one way ANOVA prior to a 2-tailed t test ; *p<0.05, **p<0.01, in comparison with corresponding vehicle control group.

3.3.4. Effect of paracetamol exposure in utero on expression of epigenetic regulatory genes in fetal rat gonads

Following previous studies in our laboratory showing inter-generational consequences of in utero paracetamol exposure (Dean et al., 2016), it was hypothesized that exposure to paracetamol or indomethacin might interfere with the epigenetic machinery of the fetal gonads. My studies focused on DNA and histone methylation. Thus, the expression of *Tet1,* which plays a role in DNA and histone methylation, was studied, as was *Ezh2* which is the enzymatic component of the polycomb repressive group 2 (PRC2) complex, which is responsible for the repressive H3K27me3 histone methylation mark. Expression of the DNA methyltransferases *Dnmt3a* and *Dnmt3b* was also determined.

The expression *of Tet1, Ezh2, Dnmt3a and Dnmt3b* in e15.5 and e17.5 testes and ovaries of fetuses exposed in utero to paracetamol or indomethacin was investigated. However, only the effect of paracetamol exposure on the e17.5 ovary could be investigated, because no indomethacin-exposed ovarian samples were available for e15.5 females. Paracetamol and indomethacin exposure resulted in increased expression of *Tet1* in both the fetal testis (e15.5 and e17.5) and ovary (e15.5), while *Ezh2* expression was increased only in e15.5 ovaries and e17.5 testes (Figures 3.7 and 3.8). As one potential analgesic-induced consequence of such altered expression was increased activity of the PRC2 complex, other mechanisms involved with PRC2 and H3K27me3 were included, such as the direct methylation and demethylation of H3K27me3 by Lysine-specific histone demethylase 1 (Lsd1) and Jumonji domain-containing protein 3 (Jjmjd3) respectively, in e17.5 testes. However, expression of neither gene was modified by exposure to paracetamol or indomethacin (Figure 3.7). In light of previous studies suggesting that PGs can affect other epigenetic regulators, such as Dnmts (Venza et al., 2012), the expression of Dnmt3s was studied. Thus, expression of *Dnmt3a*, but not *Dnmt3b,* was reduced by paracetamol exposure in e15.5 ovaries (Figure 3.8).

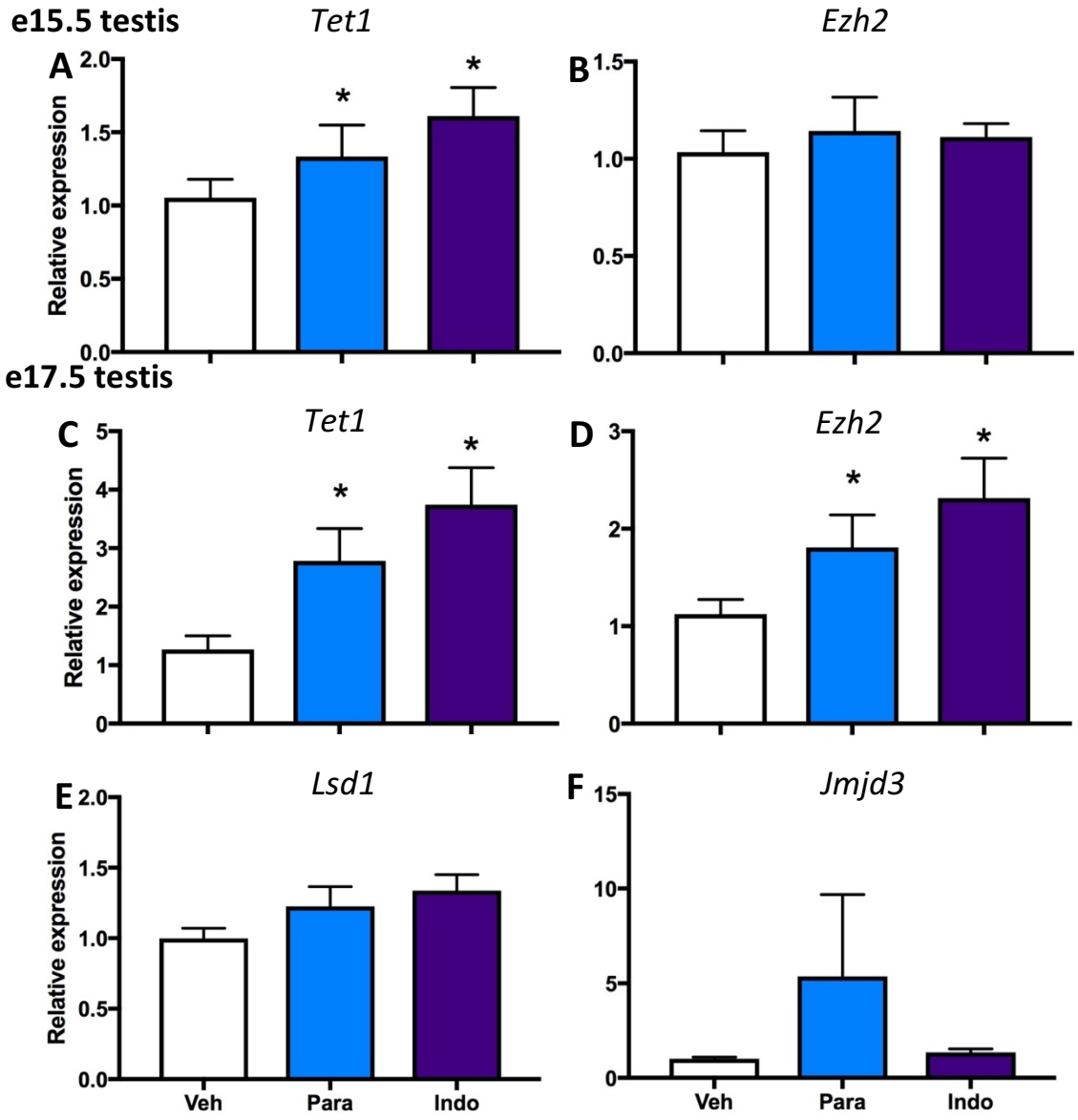


Figure 3.7. Effect of in utero exposure of pregnant rats to paracetamol (Para) or indomethacin (Indo) on mRNA expression of epigenetic regulators in the e15.5 and e17.5 testis. Pregnant rats were administered vehicle (Veh; White), paracetamol (Para; 350mg/kg/day x1; blue) or indomethacin (Indo; 0.8mg/kg/day x1; purple) commencing on e13.5, and fetal gonadal tissue was collected on e15.5 (Top) and e17.5 (Bottom), 3 hours after the final maternal treatment. mRNA expression is expressed relative to the vehicle cDNA for the same genes after the treatment exposure (Means ± SEM n=8-12 for e15.5 testis and n=11-13 for e17.5 testis). Data were analysed by one way ANOVA prior to a 2-tailed t test; *$p<0.05$, in comparison with corresponding vehicle control group.

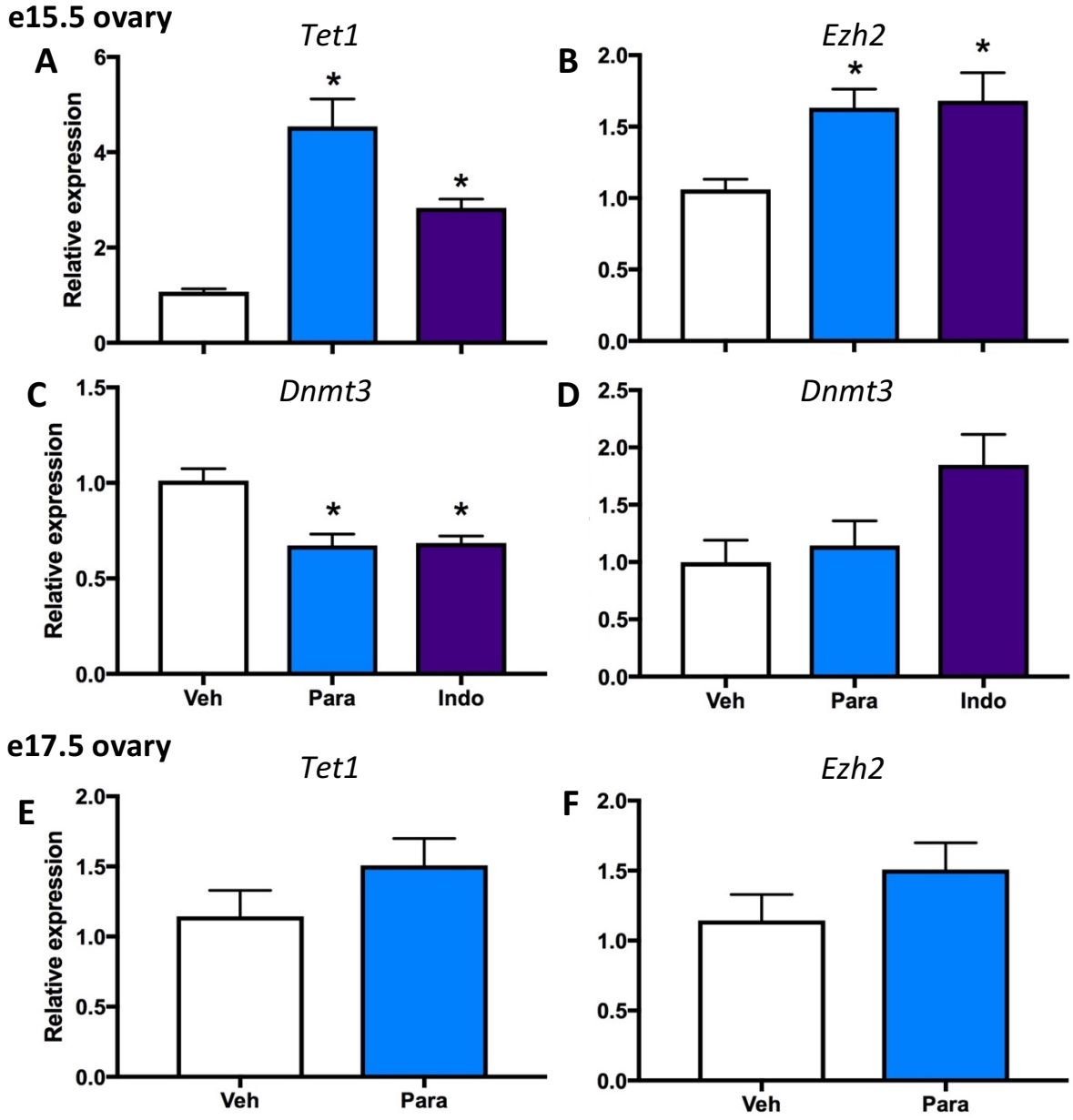


Figure 3.8. Effect of in utero exposure of pregnant rats to paracetamol (Para) or indomethacin (Indo) on mRNA expression of epigenetic regulators in fetal e15.5 and e17.5 ovary. Pregnant rats were administered vehicle (Veh; White), paracetamol (Para; 350mg/kg/day x1; blue) or indomethacin (Indo; 0.8mg/kg/day x1; purple) commencing on e13.5, and fetal gonadal tissue was collected on e15.5 (Top) and e17.5 (Bottom), 3 hours after the final maternal treatment. mRNA expression is expressed relative to the vehicle cDNA for the same genes after the treatment exposure. (Means ± SEM n=7-25 for e15.5 ovary and n=11 for e17.5 ovary). Data were

analysed by one way ANOVA prior to a 2-tailed t test; *p<0.05, in comparison with corresponding vehicle control group.

3.3.5. Elucidating the analgesic mechanism of action: specificity proteins

Indomethacin and paracetamol can both act via the PG signaling pathways (Hecken et al., 2000; Anderson, 2008; Aminoshariae & Khan, 2015), including in the fetal rat ovary in which effects on fetal GC number are found (Dean et al., 2016). Hence, the effect of these analgesics on PG downstream messengers, such as the specificity proteins (Sp) were studied. Once PGs have activated their receptors, a cascade of factors control Sp, which are translocated to the nucleus and act as expression regulators (Li & Davie, 2010). The expression of *Sp1* and *Sp2* was significantly reduced in e17.5 fetal testis after in utero exposure to indomethacin or paracetamol (Sp1 only) (Figure 3.9). Sp1 translocation to the nucleus is important for its activity and thus, it was also studied at the protein level by fluorescence immunohistochemistry on e17.5 rat ovaries. However, no difference nor in Sp1 quantity or localization was found with any of the treatments (Figure 3.10).

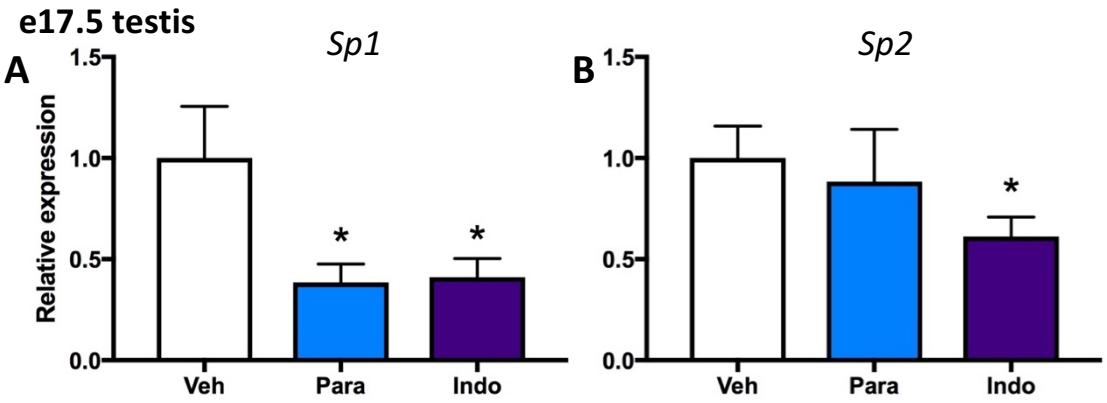


Figure 3.9. Effect of in utero exposure of pregnant rats to paracetamol (Para) or indomethacin (Indo) on mRNA expression of specificity proteins (Sp1, Sp2) in e17.5 testes. Pregnant rats were administered vehicle (Veh; White), paracetamol (Para; 350mg/kg/day x1; blue) or indomethacin (Indo; 0.8mg/kg/day x1; purple) commencing on e13.5, and fetal gonadal tissue was collected on e17.5, 3 hours after the final maternal treatment. mRNA expression is expressed relative to the vehicle cDNA for the same genes after the treatment exposure. (Means ± SEM n=11-13). Data were analysed by one way ANOVA prior to a 2-tailed t test; *$p<0.05$, in comparison with corresponding vehicle control group.

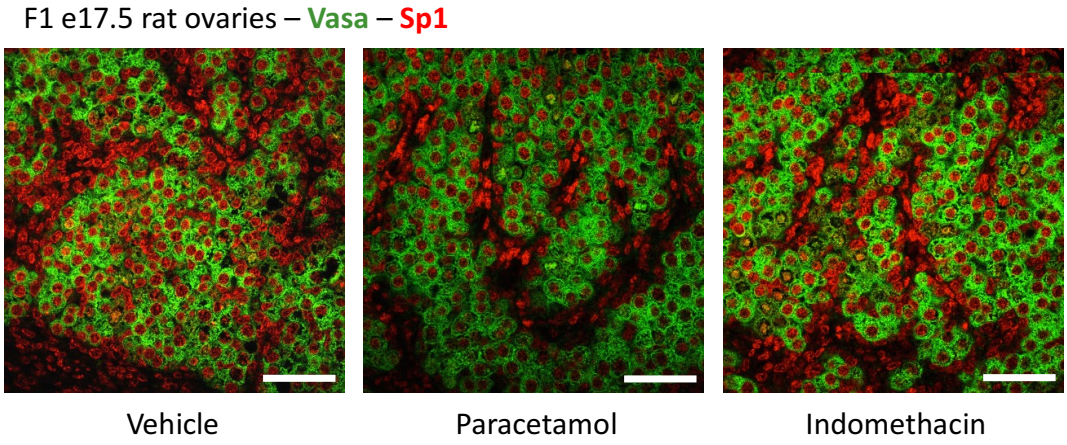


Figure 3.10. Effect of in utero exposure to analgesics on SP1 localization in e17.5 fetal rat ovary. Pregnant rats were administered vehicle, paracetamol (350mg/kg/day once daily;) or indomethacin (0.8mg/kg/day once daily) commencing on e13.5, and fetal gonadal tissue was collected on e17.5, 3 hours after the final maternal treatment. Tissue was immunostained for Vasa (GC - green) and β-catenin (red). Scale bar, 20µm.

3.3.6. Rat fetal gonad culture

½ and ¼ pieces both showed minimal necrosis and appeared viable. However, ½ gonad pieces showed a higher decrease in GC number after culture compared with ¼ pieces, so the latter was chosen as the culture standard (Figure 3.11). Previous experiments in the lab had used 48h rat fetal gonads cultures and so, this length was decided to be the main choice. Nevertheless, rat fetal testis cultures lasting 6 and 10 days were also tried. These cultures showed necrosis towards the centre of the cultures and hence, no more experiments were carried out with this culture length (Figure 3.12). Therefore, it was decided to culture and treat ¼ pieces of testis and ovary for 48h.

Previous studies on rodent fetal ovaries showed that culture produces a slight delay in normal development, estimated to be about half a day in a 48h culture (Livera et al., 2000). The expression of Dmrt1 and Oct4 was studied in cultured ovaries and testes respectively in order to track the efficiency of gonad culture. Oct4 is expressed in rat fetal testis GC during e15.5 but is downregulated by e17.5 (Culty, 2009). Similarly, Dmrt1 is expressed in e15.5 rat fetal ovary GC but disappears from the gonad by e17.5 onwards (Dean et al., 2016; Jobling et al., 2011). As expected, e15.5 control testes showed expression of Oct4 in the GCs, but this was absent in e17.5 control testes. 48h cultured testes showed no expression of Oct4, similar to the age-matched controls (Figure 3.11). In the case of the ovaries, age-matched controls showed that Dmrt1 was immunoexpressed in e15.5 samples, but was downregulated in e17.5 samples. Ovaries cultured for 48h showed some immunoexpression of Dmrt1, but there were fewer $Dmrt1^{+}$ GC than in the e15.5 non-cultured control (Figure 3.11).

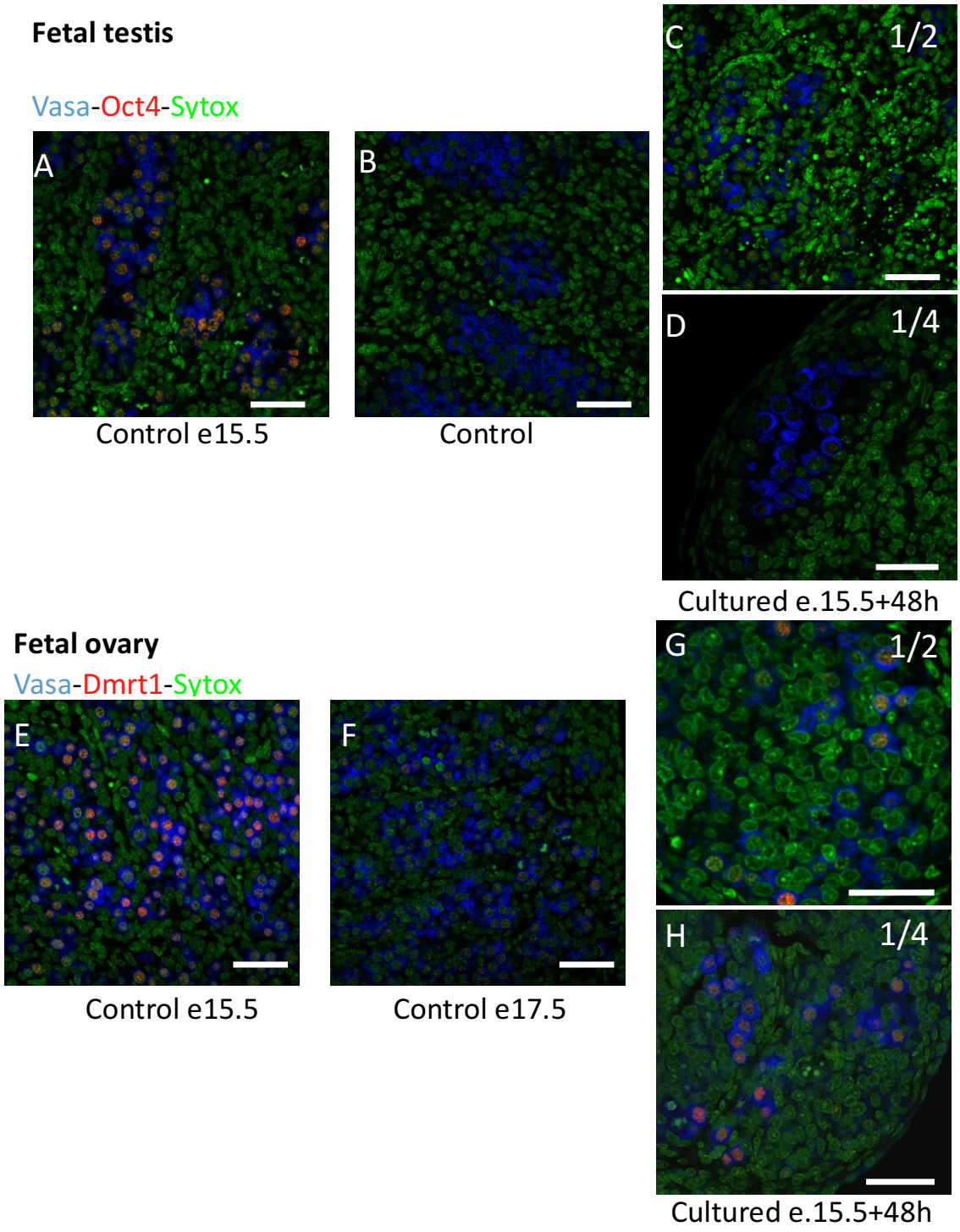


Figure 3.11. Effect of 48h culture on time-dependant development of e15.5 rat fetal gonads. ½ pieces (**C** and **G**) and ¼ (**D** and **H**) pieces of fetal rat e15.5 testes (Top) and ovaries (Bottom) were cultured for 48h in media. Panels **A, B, E** and **F** display age-match e15.5 and e17.5 testis and ovary controls Tissue was immunostained for Vasa (GC – blue) and Oct4 (Testis; red) or Dmrt1 (Ovary; red).
Tissue was counterstained with Sytox green as well. Scale bar, 20µm.

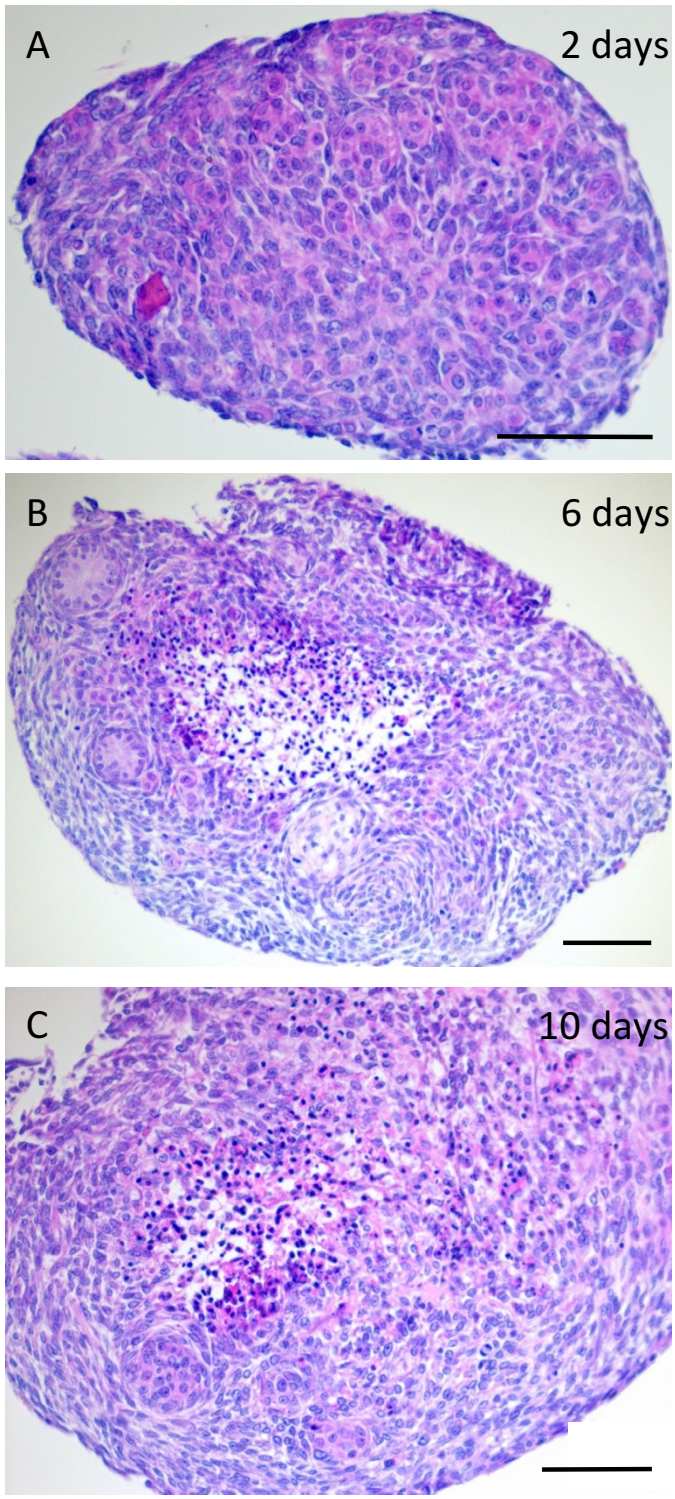


Figure 3.12. Optimization of rat testis cultures with different culture lengths. Pieces of fetal rat testis were cultured during 2, 6 or 10 days, showed in panels A, B and C respectively. Pieces were fixed and stained by H&E to detect nuclei and tissue structure. Scale bar 50µm.

3.3.7. Analgesic exposure causes changes in expression of epigenetic regulatory genes in fetal rat gonad cultures

Once the cultures were optimized and proved to be a viable model, I studied if analgesic exposure of the fetal rat gonad cultures could mimic the modifications to epigenetic regulatory gene (*Tet1*, *Ezh2)* expression seen in vivo (Section 3.3.4). Similar to the in vivo exposure, *Tet1* and *Ezh2* expression were both significantly increased in cultured ovaries and testes after 48h exposure to paracetamol or ibuprofen (Figure 3.13).

3.3.8. Effect of prostaglandin E_2 antagonists on fetal rat gonad cultures

Paracetamol and ibuprofen can both act via the PG signalling pathways (Hecken et al., 2000; Anderson, 2008; Aminoshariae & Khan, 2015). Therefore, as well as investigating the effects of paracetamol and ibuprofen on rat fetal gonad cultures, I also investigated if blockade of PGE_2 action via combined exposure to an EP2 (PF-04418948, 10μM) and an EP4 (L-161,982, 10μM) receptor antagonist, was able to induce similar effects to paracetamol and ibuprofen. *Tet1* expression was significantly increased after exposure to the EP antagonists in both cultured rat ovaries and testes, whilst *Ezh2* expression was unmodified in testes and slightly reduced in rat ovaries (Figure 3.13). Dnmts were also studied to see if decreased expression similar to the in vivo model was found. *Dnmt3a* and *Dnmt3b* expression were both reduced in cultured rat ovaries, but only Dnmt3b was significantly reduced in cultured testes (Figure 3.13).

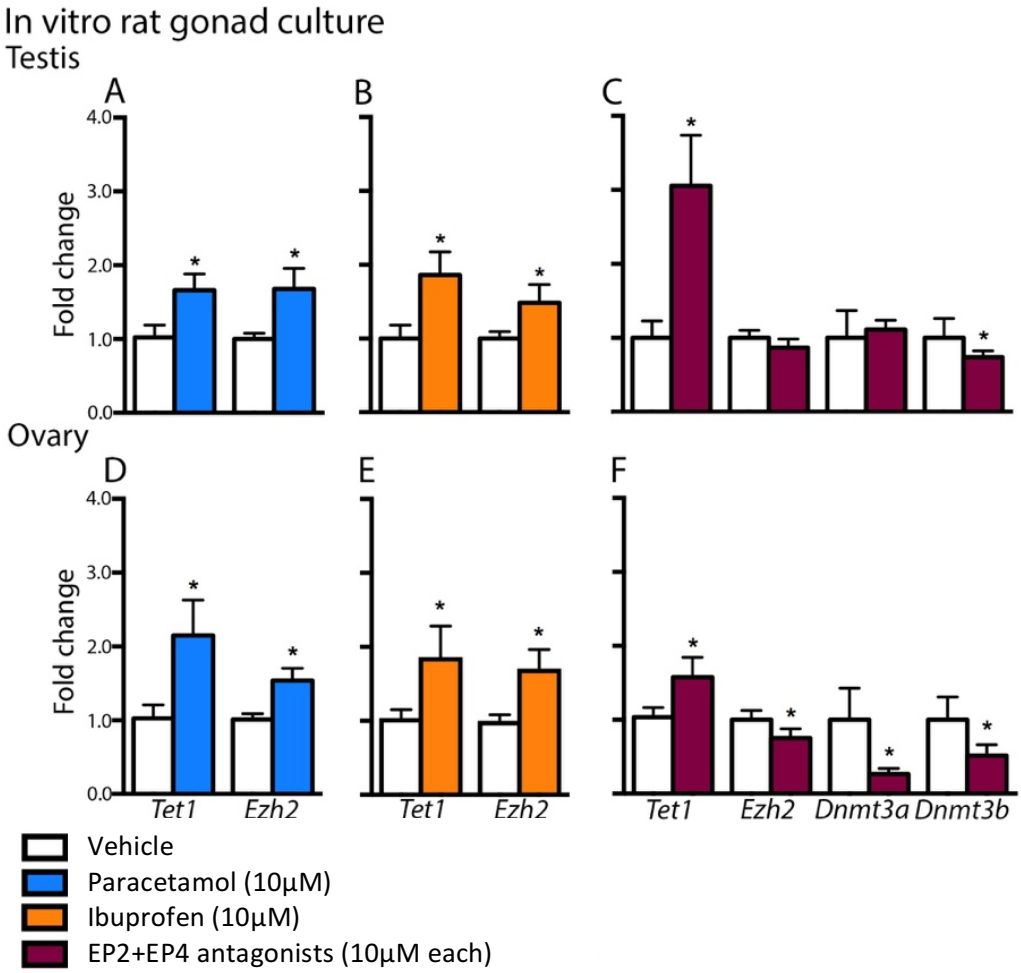


Figure 3.13. Effect of exposure of rat fetal gonad cultures to analgesics or prostaglandin E_2 antagonists on mRNA expression of epigenetic regulatory genes. Testis and ovary tissue pieces (¼) from e15.5 fetal rats were cultured for 48h with either vehicle (White, Paracetamol (blue; 10µM), ibuprofen (orange; 10µM) or EP2+EP4 antagonists [Maroon; 10µM L-161,982 (EP2 antagonist) + 10µM PF04418948 EP4 antagonist)]. Panels **A** to **F** show mRNA expression relative to the vehicle complementary DNA for *Tet1*, *Ezh2*, *Dnmt3a* and *Dnmt3b* for cultured fetal testes (**A** to **C**) or ovaries (**D** to **F**) (Mean ±SEM, n=13-30). Data were analysed by paired t-test; *p<0.05, in comparison with corresponding vehicle control group.

3.4. Discussion

3.4.1. Effect of in vivo exposure of rat fetal gonads to analgesics on the retinoic acid pathway

Retinoic acid (RA) is one of the most well-known factors participating in fetal development. It plays roles in some of the most important events in the development of the functional fetus, such as cell growth and differentiation, somatogenesis or organogenesis (Kam et al., 2012). Within the rodent gonad, RA controls the onset of GC meiosis. In the testis, RA is inactivated by its metabolizing enzyme (CYP26B1) and Stra8, a protein activated by RA involved in meiosis onset, is only activated after birth. In female mice, RA participates in meiosis onset at around e13.5 due, in part to Stra8 activity (Zhou et al., 2008a; Le Bouffant et al., 2010; Baltus et al., 2006). The timing of RA presence in the testis is key to normal GC development. Transgenic mice with a CYP26B1 knockout (i.e increased RA levels) present with GC entering meiosis prematurely, which were then arrested at the pachytene phase and exhibited increased apoptosis (MacLean et al., 2007). Previous experiments in our lab on the rat fetal ovary showed that Stra8 is down-regulated in fetal rat ovaries between e17.5 and e18.5, but is up-regulated by analgesics at these time points, suggesting an accelerated onset of meiosis (Dean et al., 2016). Modification of meiosis initiation could be one explanation for the phenotype seen, namely a reduction in GC number after exposure to analgesics (Dean et al., 2016; Holm et al., 2016).

The study of the effect of paracetamol and indomethacin on RA was done indirectly by showing their ability to up-regulate the expression of the RA synthesizers *Aldh1a1* and *Aldh1a2* in e17.5 rat fetal testes after analgesic exposure in utero. During human fetal life, *Aldh1a1* increases according to gestational age in the testis, while the contrary happens in the ovary (Childs et al., 2011). *Aldh1a2* expression does not follow the same pattern and it is decreased in older gestational ages in the testis, although it does not follow a clear pattern in the ovary (Childs et al., 2011). Decreased Aldh1a1 has been related to a decrease in RA levels leading to a delay in the onset of

meiosis (Bowles et al., 2016). There are no models of increased *Aldh1a1* expression, however this gene is increased in human gonads when meiosis starts, providing a source of RA (Le Bouffant et al., 2010). The increase in *Aldh1a1* seen in e17.5 rat fetal testes exposed to paracetamol or indomethacin in vivo could produce an increase in RA and hence cause an advance in entry to meiosis. Increased RA is normally linked with an increased expression of its receptors (Rarα, Rarβ, Rxrα, Rarγ and Rxrγ) and metabolizers (Cyp26b1) (Childs et al., 2011). However, in these studies expression of these receptors showed no change after in vivo exposure to paracetamol or indomethacin in e17.5 fetal testes. With all these results together, the increase in the levels of RA by paracetamol or indomethacin in e17.5 rat fetal testes cannot be confirmed. However, some of the results do raise the possibility. A deeper analysis and study could be addressed with models involving the use of lacZ-coupled transgenes controlled by an RA response element (RARE) (Rossant et al., 1991).

3.4.2. Effect of in vivo exposure of rat fetal gonads to analgesics on expression of genes important in gonad development

In chapter 1, I explained in detail the different pathways that the bipotential gonad can undergo to further develop into a testis or an ovary. There are two alternative pathways of sex determination depending on expression of the *Sry* gene, the role of which is to activate Sox9 expression (She & Yang, 2016). The increased expression of *Dact1* seen in e17.5 rat fetal testes exposed in vivo to paracetamol or indomethacin was the first indication of a possible interaction with the Wnt4/β-catenin pathway. Further investigation in the same model revealed a decrease in the expression of *Wnt4*. Wnt4, Rspo1 and β-catenin work together to actively inhibit *Sox9* expression in females (Elzaiat et al., 2016). In females, a decrease in Wnt4 could interfere with normal gonad development by not suppressing *Sox9* expression, resulting in trans-differentiation of granulosa cells into Sertoli cells (Lavery et al., 2012). However, it is important to recall that the analyses done in this research were only performed in

testes. Wnt4 is still expressed in the testis at low levels and different experiments have shown that complete inhibition/ablation of Wnt4 in the testis can also be detrimental for testis formation, including decreased proliferation and increased apoptosis of GC (Das et al., 2013; Boyer et al., 2012). Furthermore, if we presume that the same mechanisms affected in the testis by paracetamol or indomethacin might be affected in the ovaries, a decrease in Wnt4 could produce a more marked effect on ovary development.

β-catenin, the role of which is tightly linked with Wnt4 was also studied. Its gene expression was not modified by in vivo paracetamol or indomethacin exposure in e17.5 testes, although this was partially expected, as β-catenin action is normally studied by localisation. Fluorescence immunohistochemistry studies did not show any difference in β-catenin localization in rat e17.5 fetal ovaries after analgesic exposure. However, previous publications on fetal gonads reported difficulties of studying β-catenin localization, as its nuclear translocation is a dynamic process difficult to monitor by immunohistochemistry (Liu et al., 2009).

To make a deeper study with more possible interactions within the same sex determination system, the gene expression of *Rspo-1, Sox9, Amh* and *Gata-4* was also studied. All these genes are involved in the same pathway of sex determination, as was explained in section 1.3.2.1. Rspo1's role is tightly involved with Wnt4 and knockdown of Rspo1 or Wnt4 results in similar phenotypes including the ovarian trans-differentiation of granulosa cells into Sertoli cells (Tomizuka et al., 2008; Lavery et al., 2012). A similar phenotype is seen with a knockdown in Sox9, although in this case the sex reversal is complete (Koopman et al., 1991; Vidal et al., 2001). In the case of Amh, reduced fetal levels result in males with ectopic female reproductive tracts (Belville et al., 1999). On the other hand, higher expression of *Amh* in female mice produces regression of the müllerian ducts (Behringer et al., 1990). Gata-4 is needed during early gonad development for an appropriate *Sry* expression and hence

for the proper development of the testis and somatic cell differentiation (Bielinska et al., 2007). However, the study of Rspo-1 Sox9, Amh and Gata-4 did not show any significance difference or pattern when studied in e17.5 rat fetal testes exposed in vivo to paracetamol or indomethacin. This could mean that paracetamol and indomethacin are acting on a really specific part of the gonad differentiation pathway, but not all players are involved, or potentially that the other players might be altered, but not their gene expression. For example, β-catenin knockdown mice showed decreased expression of *Wnt4*, but no variation in the expression of *Rspo-1* (Liu et al., 2009). In a similar way, the decreased expression of *Dact-1*/3 seen after analgesic exposure in e17.5 rat testes could interact with the Wnt4/β-catenin pathway and not modify *Rspo-1* or expression of the other genes in this pathway.

3.4.3. Effect of in vivo exposure to analgesics on GC development in fetal rat gonads and on the follicle pool

Previous experiments in the lab have shown that one of the effects of analgesic exposure on rat fetal ovaries is a decrease in GC number, as well as decreased adult ovary weight (Dean et al., 2016). The creation of an oocyte pool in the ovary is a complex process and modifications to any of the mechanisms involved can cause a reduction in GC/oocytes. This reduction can arise for several reasons, including decreased proliferation (resulting in a smaller maximum oocyte pool), or increased atresia (natural oocyte death). Foxo3, Notch2, Epiregulin (and its receptor EGFR), and inhibin alpha, all of which are involved in the development of GC and which can affect the follicle pool (John et al., 2008; Xu & Gridley, 2013; Feng et al., 2014b) were studied. As mentioned above, only e17.5 fetal rat testes were available for study. However these genes are also expressed in testes and the mechanisms that regulate them are potentially similar in both the testis and ovary. The expression of *Foxo3, Notch2, Epiregulin, EGFR and Inha* were significantly increased after in vivo exposure of paracetamol or indomethacin in the e17.5 testis. The relevance of these modifications in gene expression is difficult to address. Variations in the expression

of all of them have consequences for fetal gonad development. Foxo3 has been proposed as a preserver of the follicle pool (Pelosi et al., 2013) and different studies show how knockdown of *Foxo3* develops into an ovarian burn-out (early loss of oocytes) (Castrillon et al., 2003), while up-regulation delays follicle development (Pelosi et al., 2013). Notch2 is involved in the initiation of oocyte nest breakdown and follicle formation (Xu & Gridley, 2013) and its knockdown shows decreased follicle formation and abnormal follicular growth (Trombly et al., 2009). Furthermore, in males, *Notch2* expression is directly related to testosterone levels via regulation of Leydig cells. (Defalco et al., 2013). Epiregulin is involved in bidirectional signalling between somatic and germ cells and it is a potent stimulator of oocyte maturation and cumulus expansion (Hsieh et al., 2007; Romero & Smitz, 2010). Inha is one of the subunits of inhibin and activin, glycoproteins that are part of the transforming growth factor superfamily. Upregulation of *Inha* expression in fetal ovary is associated with impaired fetal ovarian folliculogenesis (Billiar et al., 2003). Loss of Inha uncouples oocyte-granulosa cell dynamics and disrupts postnatal folliculogenesis (Myers et al., 2009). The variations in the expression of these genes seen after analgesic exposure in the e17.5 fetal rat testis suggest that similar effects can potentially occur in the analgesic exposed rat ovary. I was unable to address this possibility directly because of a lack of available tissue, but in theory such effects could be related to the loss of GC seen in previous experiments in the lab (Dean et al. 2016)

Furthermore, I decided to study Dmrt1, which is tightly related to RA via its repression of *Stra8* expression. Modifications in the expression of *Dmrt1* have previously been shown to modify the normal onset of meiosis in male and female mice and hence to affect normal GC development (Krentz et al., 2011; Matson et al., 2011). However, neither of the analgesics were able to modify *Dmrt1* expression on in vivo exposed e17.5 fetal rat testis.

Further studies should be undertaken in order to determine the actual consequences of paracetamol and indomethacin effect on these gene regulators of gonad development, including the study on ovary and on other time points. All the analyses shown in this section are from studies on RNA expression. Further studies on the protein level could reveal a more biologically relevant consequence of the regulators of gonad development as a result of analgesic exposure. Furthermore, the phenotype of GC loss could be more driven by one of the factors or by a combination of all of them, and also it was not possible to address if the modifications in gene expression were a cause or consequences of the other changes.

3.4.4. Rat fetal gonad cultures as a viable model

The exposure of pregnant rats to analgesics is a model where it is possible to study the effects of these analgesics in a physiological manner. Nonetheless, it has the disadvantage of not knowing if the phenotypes seen are a result of direct or indirect effects of the treatment. The ability of common analgesics to cross the placenta has been reported in several studies (Nitsche et al., 2016; Alano et al., 2001; Ma et al., 1989; Weigand et al., 1984). Still, it is difficult to discern between the actual effects as a result of the analgesics themselves, or the effects that could be caused because of an alteration in the mother´s physiology that could be transmitted to the fetus. One way to to separate these indirect effects is by eliminating the mother from the equation and exposing the fetal gonads directly to the analgesics.

The analysis of the whole gonad cultures showed necrotic areas in the interior of the testes and ovaries, probably because of the limited diffusion of nutrients and gasses. Regarding the smaller pieces, cultures with ½ and ¼ pieces showed viable cultures without prominent areas of necrosis. However, comparing the number of GC and the appearance of the cultures, ¼ pieces as my model of study were used. In terms of the length of the culture, short term exposure (48h) was our priority, although longer exposures would allow us to modify the system in different ways. However, 6 and 10

days exposure on testis cultures did not develop into usable cultures as the middle part of the pieces showed necrosis. This is possible because of the fast metabolism of the rat cells compared with human and the need for extra supplies in the tissue that is not in contact with the external media.

Another studied aspect of the cultures was if they exhibited normal development. Previous studies on mice fetal ovaries showed how cultured gonads suffered from a small delay, which was estimated at half a day in a 48h culture (Livera et al., 2000). Dmrt1 and Oct4 immunoexpression were used in ovaries and testes respectively to study if they continued normal development. Oct4 in testis and Dmrt1 in ovaries are normally expressed at e15.5 and are downregulated by e17.5 in fetal rat GCs (Culty, 2009; Dean et al., 2016; Jobling et al., 2011). The reduction in Oct4 immunoexpression in 48h fetal rat testis cultures, which was similarly to the age-match controlled, confirmed that there was good testis development in the hanging drops. In the case of the fetal ovaries, the result was not as clear, because, even if reduced, there was still some expression of Dmrt1 in e17.5 cultured ovaries whereas immunoexpression had switched off in the age matched controls. This is a signal of a delay in ovary development in the hanging drops.

3.4.6. Analgesics are capable of modifying the epigenetic machinery of the fetal rat gonads

Results on the gene expression of epigenetic regulators in the in vivo exposed fetal rat gonads and the in vitro rat fetal gonad cultures showed for the first time that paracetamol, indomethacin and ibuprofen are able to modify the expression of *Tet1* and *Ezh2*, which suggests a possible modification of the PRC2 complex. The most studied role of Tet1 is in the conversion of 5-mC into 5-hmC (Yamaguchi et al., 2013). Yet, the DNA is highly demethylated during the stages of development studied in this paper (e13.5-e17.5) (Guo et al., 2014; Rose et al., 2014) and therefore, more emphasis on the secondary and less studied role of Tet1 in the recruitment of the

PRC2 complex over specific histone methylation points was made. Alterations of the PRC2 complex can result in a H3K27me3 increase, a known marker of gene repression (Wu et al., 2011). *Tet1* and *Ezh2* gene expression were increased after analgesic exposure in in vitro fetal gonad cultures and in e15.5-e17.5 gonads exposed in vivo to paracetamol in both sexes, showing a strong pattern of similarity. Genes targeted by the PRC2 complex are specific for cell type and species (Simon & Kingston, 2009) and hence, genes regulated by the PRC2 complex (and H3K27me3) in fetal gonads should be addressed in future research.

Previous publications have shown the ability of PGE_2 to affect Dnmts (Venza, et al. 2012), and hence I decided to study other regulators of the epigenetic machinery related to DNA methylation, such as Dnmt3a and Dnmt3b. Both of these enzymes showed decreased mRNA expression in the in vivo and in vitro systems after analgesic exposure, although the results were not completely consistent among all the models. Unlike in somatic cells, DNA within GCs is highly unmethylated during the majority of fetal development and it is only remethylated after birth in the case of females, and during the prespermatogonial stage in males (Reik, 2001). Therefore, GCs in the stages here studied are highly unmethylated. However, some publications have shown that some genes are protected from global demethylation (Guo et al., 2014; Smith et al., 2014). The consequences of a decreased expression of Dnmts on fetal gonads during gestation is largely unknown. However, this could affect the infrequent methylated regions still present in the GC genome in this period and hence, their gene expression.

3.4.6. Potential mechanistic pathways affected by analgesics

One of the main aims of this research was to try to elucidate the actual mechanism of action of analgesics on the fetal gonads. Several publications have shown phenotypic differences in gonads exposed to these analgesics, however, few of them have focussed on finding the actual mechanisms involved. The principal hypothesis

that my studies focussed on was that both paracetamol and ibuprofen can alter the PGE_2 pathway.

Using the in vivo exposure model, I focussed on study of the SP proteins. These are ubiquitously expressed zinc finger-containing proteins able to bind DNA to activate or repress gene transcription. They are able to regulate the expression of a wide variety of genes to control a wide variety of cellular processes, including cell growth, apoptosis or differentiation. SP are regulated by different modifications including phosphorylation, acetylation, glycosylation, ubiquitination or sumoylation (Tan & Khachigian, 2009). One of the multiple pathways involved in SP modifications is as an indirect effect of PGs, through the PI3-K/Akt (Chen et al., 2015). SP proteins are gene-specific, cell-specific and are known to regulate some gonadal genes. They have also been linked with the regulation of DNMTs and some histone modifications (Huang et al., 2012), which correlates with the effects showed on the epigenetic regulators by paracetamol, indomethacin and ibuprofen after in vivo exposure of fetal gonads and after exposure in vitro of fetal gonad cultures (Sections 3.3.4 and 3.3.7). The expression of SP1 and SP2 was significantly reduced after in vivo exposure to indomethacin in the e17.5 testis, but paracetamol exposure only reduced SP2 expression. SP targets depends on the type of modification that it undergoes and the cell type. There are not many studies about SP proteins in fetal gonads and, therefore, the consequences of the decrease in SP expression are unknown. For this reason, deeper analysis on the specificity of SP proteins in the fetal gonads should be addressed in future studies.

Similar to β-catenin, the regulatory abilities of SP proteins towards specific genes is controlled by their localization (Tan & Khachigian, 2009). Once SP are targeted and suffer a modification, they are translocated into the nucleus, where they target different gene promoters. When studied by Immunohistochemistry in e17.5 ovaries exposed in vivo to paracetamol or indomethacin, SP1 nuclear localization was

comparable within the different treatments. It is difficult to address if paracetamol and indomethacin, in this concentration, are able to substantially modify the localisation of SP1 in order to be seen by immunofluorescence. On the other hand, there are no previous reports of the localization of SP1 in fetal gonads, and hence it is more difficult to study the possible consequences of the analgesics on their localization.

To provide mechanistic support to my findings and study the hypothesis that paracetamol and ibuprofen effects are caused by alterations in the PGE_2 pathway, the fetal rat gonad cultures were exposed to PGE_2 receptor (EP2+EP4) antagonists. The results showed that PGE_2 receptor antagonists, when added together to the rat fetal gonad culture system, were able to show similar modifications to those seen after paracetamol and ibuprofen exposure. Examples of these are the same patterns of increase in *Tet1* and *Ezh2* expression when exposed to the analgesics or to the PGE_2 receptor antagonists. These results are in consonance with other studies relating PGE_2 with epigenetic regulators, such as DNTM3A and DNMT3B, but also histone modifications, such as H3K9me3, H3K27me3 or H3K9ac (Arosh et al., 2015; Xia et al., 2012; Venza et al., 2012). My results show comparable modifications, although it would be relevant to perform a deeper study on histone modifications in order to obtain more information regarding the exact modifications, including affected genes and related protein levels and possible further consequences of epigenetic modifications. It is important to mention that previous studies on fetal rat gonad cultured showed how the epigenetic state of these cultures is maintained during the culture (Rwigemera et al., 2017).

3.4.7. Final conclusions

We are still far from understanding the complete mechanism of action of common analgesics on the fetal gonads. Yet, the results shown in this chapter of the different experiments performed in two different rat models (in vivo and in vitro exposure),

give hints of where we should keep researching. The modifications to retinoic acid regulators, as well as to the different genes important for gonad and GC development, show some possible mechanisms affected. Whether these modifications are the cause of the phenotypes reported in previous studies, remains to be addressed. Furthermore, these modifications might be a cause or a consequence of modifications to the epigenetic regulatory genes described. Even more important, the analgesic-induced modifications to the epigenetic regulators might be the first mechanism able to explain the transgenerational effects of analgesics shown by our lab in the past (Dean et al., 2016).

The development and optimization of the in vitro fetal rat gonad cultures can be useful for future studies aimed at elucidating the mechanism of action of paracetamol and ibuprofen. Meanwhile, these cultures were able to show that this mechanism is probably mediated by PGE_2 as evidenced by the experiments using PGE_2 receptor antagonists. Further experiments using these antagonists in other systems will be explained in chapter 5.

Chapter 4: Analgesic exposure in human fetal gonads

4.1 Introduction

Among all the studies focusing on the effects of analgesic exposure during the gestational period, at the start of this thesis none of them focused on the effect of analgesics on fetal GC. However, in the last year, some studies, including one from my lab, have focused on analgesic exposure effects during pregnancy in the rat and shown how this can lead to a reduction in fetal GC number in both sexes (Dean et al., 2016; Holm et al., 2016). Experiments in this lab also showed adult consequences of fetal analgesic exposure, such as a decrease in adult ovary weight, but also reduced fertility (reduced pups per litter), probably as a consequence of the reduced oocyte number. Yet, no relevant studies have focused on fertility consequences of fetal analgesic exposure in human models. Hence, the effect of paracetamol and ibuprofen on GC in human fetal gonad tissue was studied. As explained in chapter 1, the consequences of analgesic exposure during pregnancy might depend on the gestational period (Hurtado-Gonzalez & Mitchell 2017; Kristensen et al., 2011; Snijder et al., 2012) and therefore, the possible effects were studied at different gestational time points using different models of study: hanging drop culture for 1st trimester fetal testes and ovaries and xenografts for 2nd trimester fetal testes.

In this chapter, I will describe the different experiments performed to study the effects of paracetamol and ibuprofen on fetal human gonadal GC. The relative number of GC present after analgesic exposure was studied to find possible similarities to the decrease in GC number found in previous studies performed in rats (Dean et al., 2016; Holm et al., 2016). Cell death and GC proliferation as a consequence of the different treatments was studied to find possible mechanisms to explain the decrease in GC number found. Moreover, with 2nd trimester xenografts, the effect of 7 day's exposure to a higher dose of paracetamol and the effect of a shorter exposure to paracetamol (1 day only), was performed.

4.3 Material and Methods

Experiments involving in vivo human exposure to analgesics during pregnancy are not possible for obvious ethical reasons. Therefore, an established model system was used to study human fetal exposure to analgesics, using fetal gonads obtained from elective abortions. These samples came from the Royal Infirmary Hospital (Edinburgh) and the Human Developmental Biology Resource (London and Newcastle) as detailed in section 2.3. The dissection of the fetuses was done by R. Bayne, R. Rosario in the case of Edinburgh samples and by the local team in London and Newcastle. W. Mungall, J. Henderson, and M. Dodds assisted with the animal work and tissue collection related with the xenograft studies.

Dissected testes and ovaries were exposed to 10μM paracetamol or ibuprofen for 7 days using the hanging drop culture system (Figure 2.2), where a piece of the gonad is cultured in a drop containing 30μl of medium in the cover of a petri dish containing PBS (see section 2.3.2). All samples, were fixed in Bouin´s and processed for cutting by J. Macdonald, S. MacPherson and myself. The analysis was done by multiplexed fluorescence immunohistochemistry following the protocols explained in chapter 2 (section 2.4.10) and using specific markers for GC (AP2γ and MAGE-A4), male somatic cells (SOX9) or proliferative cells (Ki67). Cells were counted, blind to the treatment, with the help of ZEN software. The different subpopulations of GC, namely gonocytes (AP2γ^+) and pre-spermatogonia (MAGE-A4$^+$) were counted in cultured testis pieces after exposure to paracetamol or ibuprofen using Zen software (section 2.5.2). GC were counted in each tissue cross-section and this number was then expressed relative to the total surface area of seminiferous tubules in those sections. This was done to correct differences in testis tissue size/composition between cultures. Two different ratios were calculated: total GC number and the ratio of the specific subpopulation of gonocytes (AP2γ^+). The results obtained were analysed by two-way ANOVA, one variable being the different gonad pieces within the same fetus (replicates) and the other factor the different human samples (n number).

1st trimester cultured ovaries were immunostained for AP2γ, to detect GCs and Hoechst as a counterstain. Fetal ovarian GCs do not express MAGE-A4 and therefore it cannot be used as a GC development marker. Another difference from the testis is that, unlike males, fetal ovaries do not develop cords. Fetal ovarian GCs are organized in nests, although these are not formed by this stage of development. Therefore, to compare AP2γ GC number, the whole gonad surface area was used as a reference point.

For 2nd trimester gonads, a more physiological model was used, xenografting gonad pieces directly under the back skin of nude mice. The protocol for xenografting was performed by W. Mungall and J. Macdonald. For the xenograft model (section 2.3.3), the host mice were exposed to paracetamol (20mg/kg/x3 daily) for 1 or 7 days or to ibuprofen (10mg/kg/x3 daily) for 7 days, using human-equivalent exposure regimens. A 7 days high dose paracetamol (350mg/kg daily) was also studied using the xenograft model. Samples were fixed and immunostained in the same way as 1st trimester fetal testis cultures. The number of AP2γ^+ and MAGE-A4$^+$ GC was then quantified in each xenograft (2-5 per fetus) and expressed relative to Sertoli cell (SOX9+) number. This was done to correct for the variability in xenograft size and composition between samples.

4.3 Results

4.3.1 Viability of human fetal gonads after culture

At the gross level, the cultured tissue pieces looked healthy with no visual signs of necrosis or apoptosis. General viability of this model was studied by analysing apoptosis using cleaved caspase 3, which showed no difference between cultures and treatments (Figure 4.1). Each testis piece from the different treatment groups showed the presence of somatic (SOX9$^+$) and GC (AP2γ^+ or MAGE-A4$^+$). Ovary pieces showed the presence of AP2γ^+ GC and somatic cells (AP2γ^- and Hoechst$^+$). There was

a general loss of GC as a consequence of the culture, independent of treatment effects, when the cultured samples were compared to the pre-culture control tissue

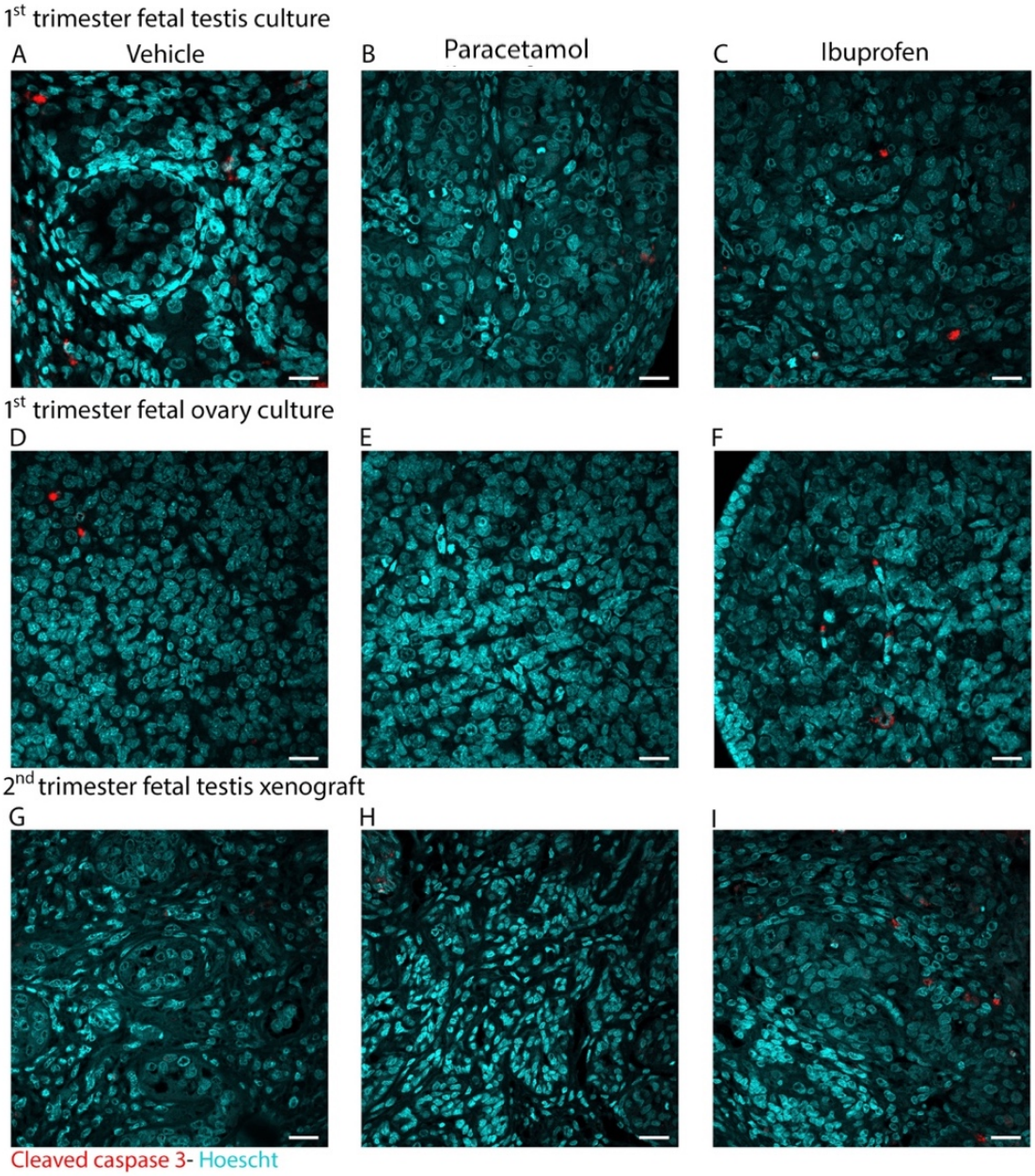


Figure 4.1. Effect of analgesic exposure on apoptosis in human fetal gonads. Apoptosis as a consequence of analgesic exposure was studied in 3 different systems: 1st trimester fetal testis (**A-C**) and ovary (**D-F**) cultures, and 2nd trimester fetal testis xenografts (**G-I**) exposed to human relevant doses of paracetamol or ibuprofen. Representative images from the tissue are shown. Tissue was immunostained for Cleaved caspase 3 (red), as an apoptosis marker and Hoechst (counterstaining - blue). Scale bar, 20µm

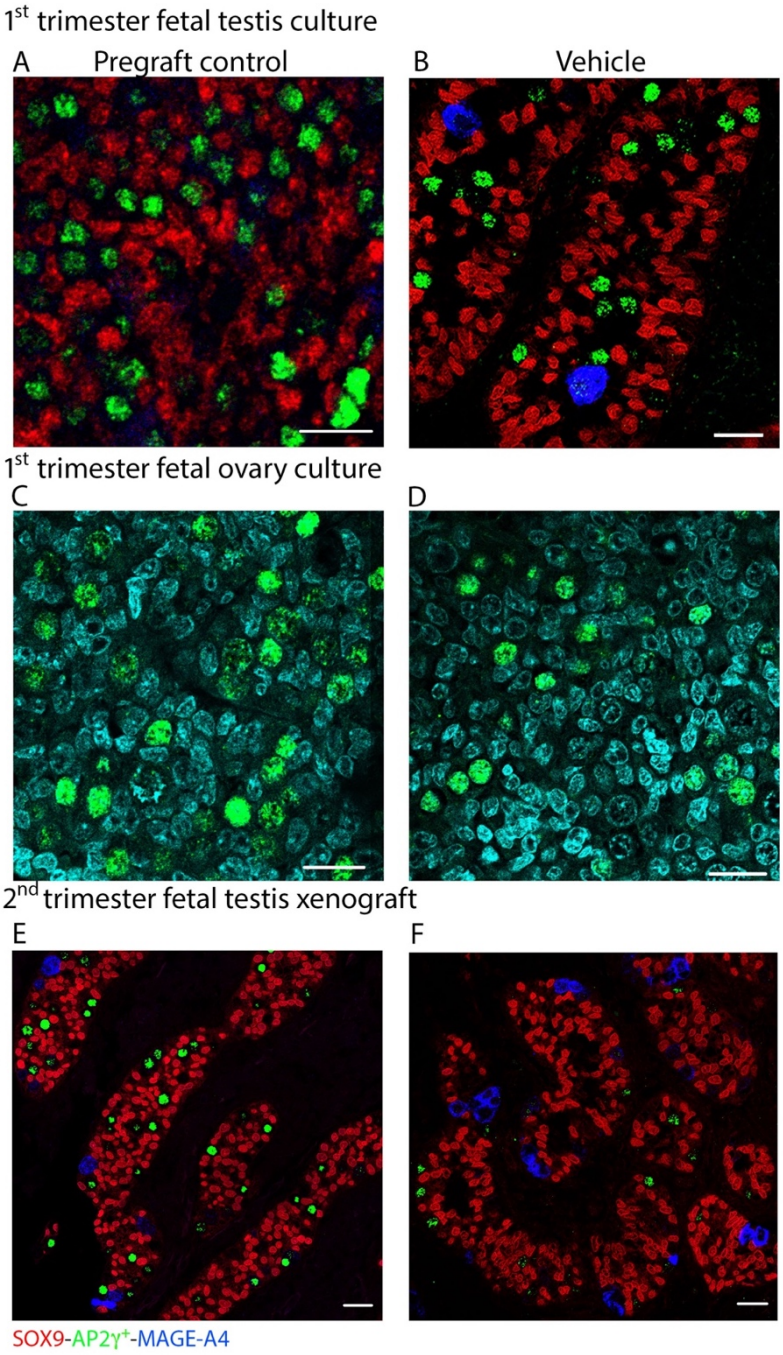


Figure 4.2. Effect of human fetal gonad culture or xenografting on GC survival. The number of GC as a consequence of human gonad culture or xenografting was studied in 3 different systems: 1st trimester fetal testis (**A** and **B**) and ovary (**C** and **D**) culture, and 2nd trimester fetal testis xenografts (**E**-**F**). Pregraft controls (**A**, **C** and **E**) were compared with vehicle (**B**, **D** and **F**). Testicular tissue was immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC - blue). Ovarian tissue was immunostained for Hoechst (counter stain - cian) and AP2γ (GC - green). Scale bar, 20µm.

4.3.3 Effect of analgesic exposure on 1st trimester testis

Paracetamol exposure significantly reduced the ratios of total GC number and gonocyte number relative to the total surface area of seminiferous tubules in those sections, by 18% ($P<0.001$) and 28% ($P<0.01$) respectively (Figure 4.3). Ibuprofen exposure similarly reduced total GC number (15%) and AP2γ^+ GC number (22%), but only the reduction in AP2γ^+ GC was statistically significant ($p<0.01$) (Figure 4.3). The number of pre-spermatogonia per testis area was not calculated because these cells were infrequent at this early stage of gonad development and, therefore, the number of cells expressing MAGE-A4 was insufficient to perform a meaningful statistical analysis.

To investigate possible explanations for the analgesic-induced decrease in GC number after paracetamol or ibuprofen exposure, I focused on increased apoptosis or reduced proliferation as possible causes. As mentioned above, apoptosis was studied by fluorescence immunohistochemistry using cleaved caspase 3, a well-known marker of apoptosis. However, the presence of apoptotic cells was minimal in all the samples studied, including samples exposed to analgesics (Figure 4.3). Therefore, proliferation was studied by co-immunostaining 1st trimester testis and ovary cultures for Ki-67 and both GC markers. Proliferative GC were defined as GC expressing Ki-67 and one of the GC markers. Cultured testis samples showed a reduction in the proportion of proliferative AP2γ^+ gonocytes after exposure to paracetamol (22% reduction; $p<0.05$) or ibuprofen (28% reduction; $p<0.05$). The total number of proliferative GC was not significantly altered however (Figure 4.3).

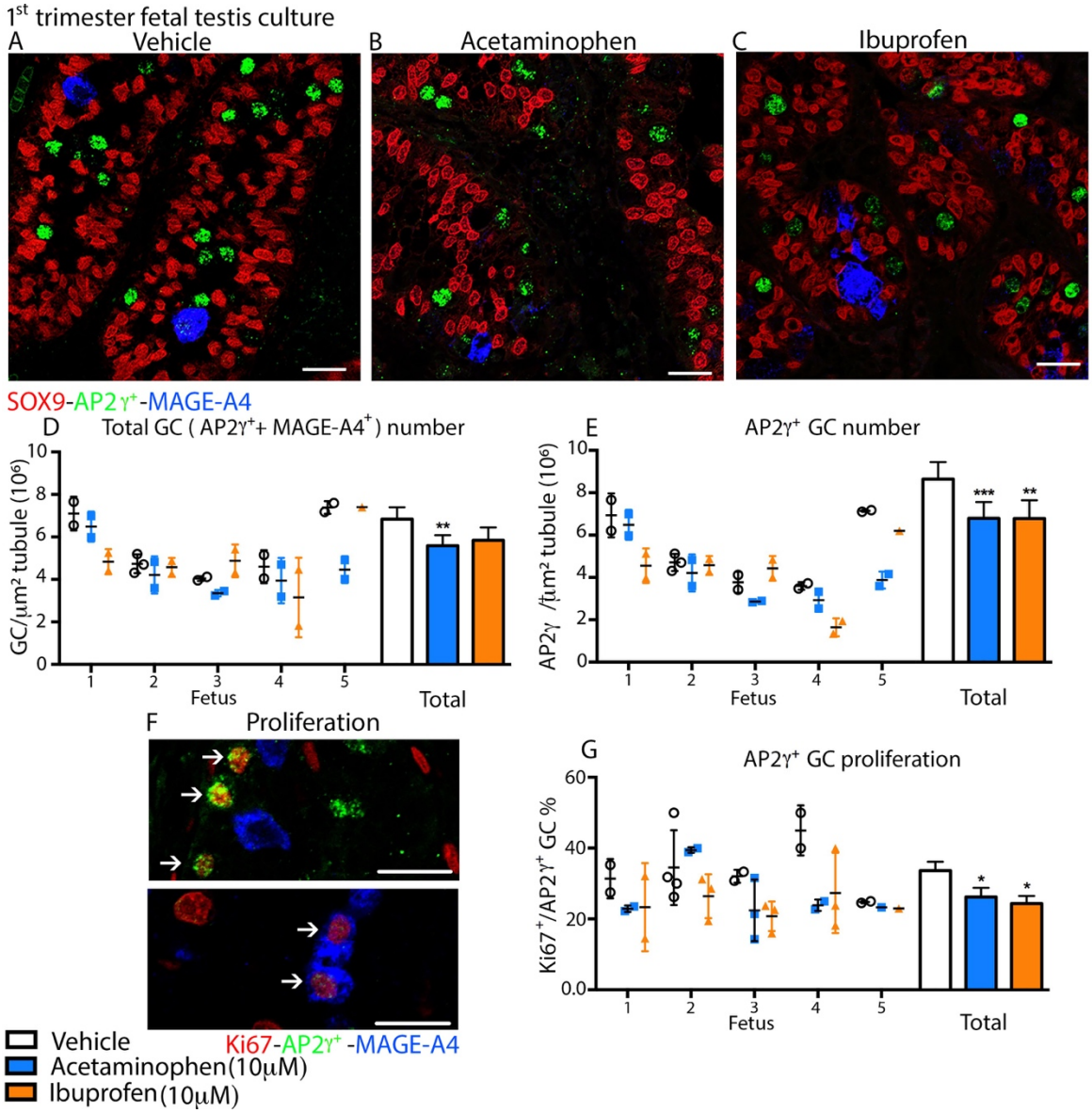
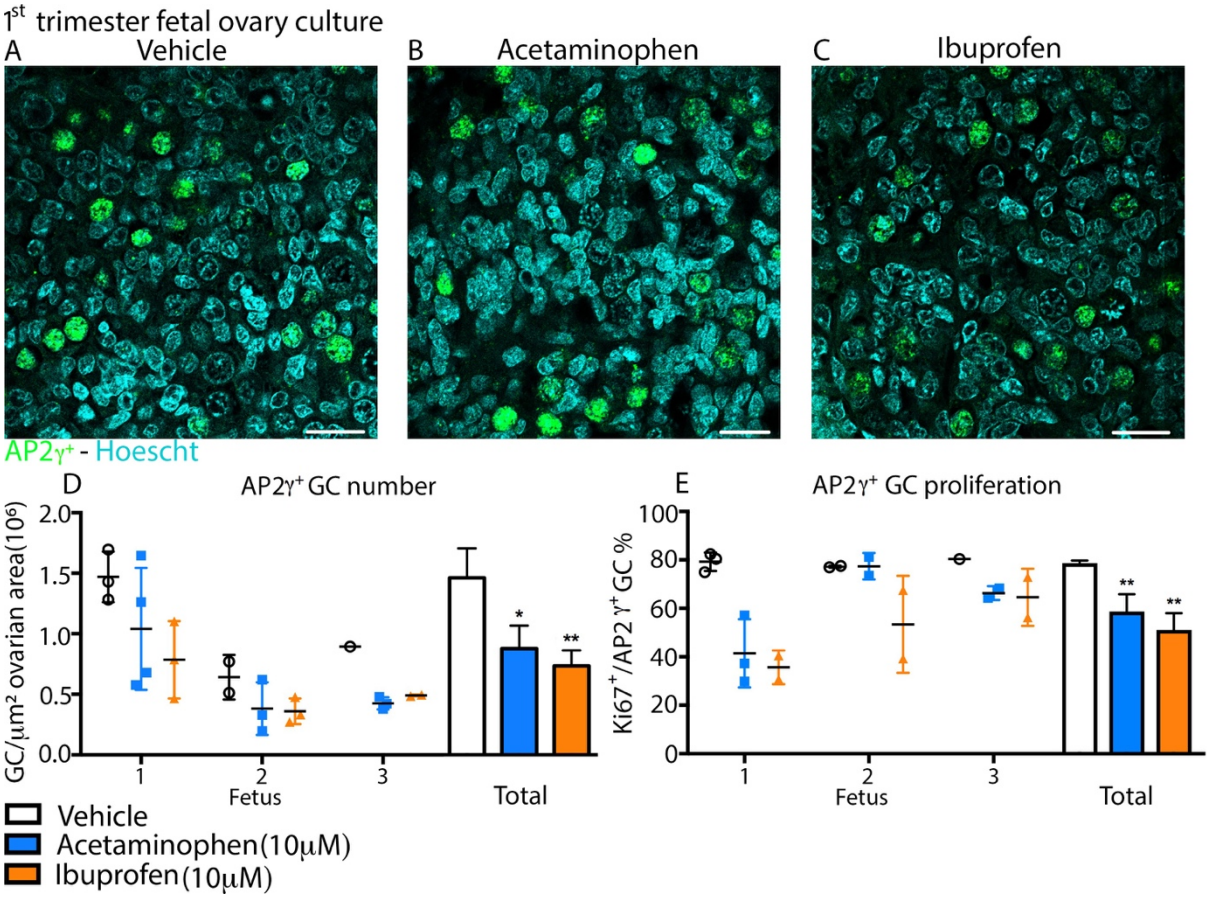


Figure 4.3. Effect of analgesic exposure of 1st trimester fetal human testis tissue for 7 days in hanging drop culture on germ cell number. Testis tissue pieces (~1mm^3) from 5 fetuses (8-11 gestational weeks) were cultured for 7 days with vehicle, paracetamol (10µM) or ibuprofen (10µM). Tissue was immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC - blue) as depicted in panels **A**-**C**. Scale bar, 20µm. Individual data points represent counts from a single tissue piece (replicates) in panels **D** and **E**. Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM; the overall mean ± SEM for each treatment group is also shown (Total). Panel **F** shows an example of a testis tissue section from a vehicle-exposed sample, triple-immunostained for the proliferation marker Ki67 (red), AP2γ (green) and MAGE-A4 (blue). White arrows refer to proliferative GC showing double staining for Ki67 and AP2γ (upper panel) or MAGE-A4 (lower panel). Quantification of the % of proliferative (Ki67$^+$) AP2γ$^+$ GC from the different treatment groups is shown in panel **G.** Data analysed by 2-factor ANOVA (*p<0.05, **p<0.01, ***p<0.001 versus corresponding vehicle-exposed group).

4.3.3 Effect of analgesic exposure on 1st trimester ovary

Similar experiments were performed on 1st trimester fetal human ovaries as for the fetal testes. Exposure of 1st trimester ovaries to either paracetamol or ibuprofen resulted in a reduction in the proportion of total AP2γ^+ GC of 43% ($p<0.05$) and 49% ($p<0.01$) respectively, when compared to the vehicle (Figure 4.4). The proportion of proliferative GC was also reduced, a 25% reduction ($P<0.01$) by paracetamol and 35% reduction ($P<0.01$) by ibuprofen.

Figure 4.4. Effect of analgesic exposure of 1st trimester fetal human ovary tissue for 7 days



in hanging drop culture on germ cell number. Ovary tissue pieces (~1mm^3) from 3 fetuses (9-11 gestational weeks) were cultured for 7 days with either vehicle, paracetamol (10µM) or ibuprofen (10µM). Tissue was fixed and double-immunostained for AP2γ (GC - green) and Hoechst (counterstaining - blue) as depicted in panels **A**-**C**. Scale bar, 20µm. Individual data points represent GC counts (AP2γ^+) from a single tissue piece (replicates) in panel **D**. Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total). Quantification of the proliferative (Ki67$^+$) AP2γ^+ GC from the different treatment groups is shown in panel **E**. Data in **D** and **E** was analysed by 2-factor ANOVA (*$p<0.05$, **$p<0.01$, versus corresponding vehicle-exposed group).

4.3.3 Effect of analgesic exposure on 2nd trimester testis xenografts

Xenografts recovered at the end of the experiment exhibited good preservation of testicular tissue structure and seminiferous cords generally with no apoptotic/necrotic areas. Samples exposed for 7 days to human relevant doses of paracetamol or ibuprofen revealed a reduction in total GC number (AP2γ^+ + MAGE-A4$^+$ GC) of 38% ($p<0.05$) and 53% ($p<0.05$) (Figure 4.5), respectively when compared with samples exposed to vehicle. The effect of the analgesics on the different GC subpopulations was also studied. The AP2γ^+ GC subpopulation was significantly reduced in number after paracetamol exposure (30% reduction, $P<0.05$). A comparable trend was also found after ibuprofen exposure, although this was not statistically significant (Figure 4.5). The MAGE-A4$^+$ GC subpopulation, quantifiable in 2nd trimester samples, was also studied. However, numbers of MAGE-A4$^+$ GC were not significantly modified by paracetamol or ibuprofen exposure (Figure 4.6). The proliferation of total GC and AP2γ^+ GC was calculated, as performed for 1st trimester hanging drop cultures (section 2.5.2), although neither of the analgesic treatments caused a significant modification (Figure 4.7).

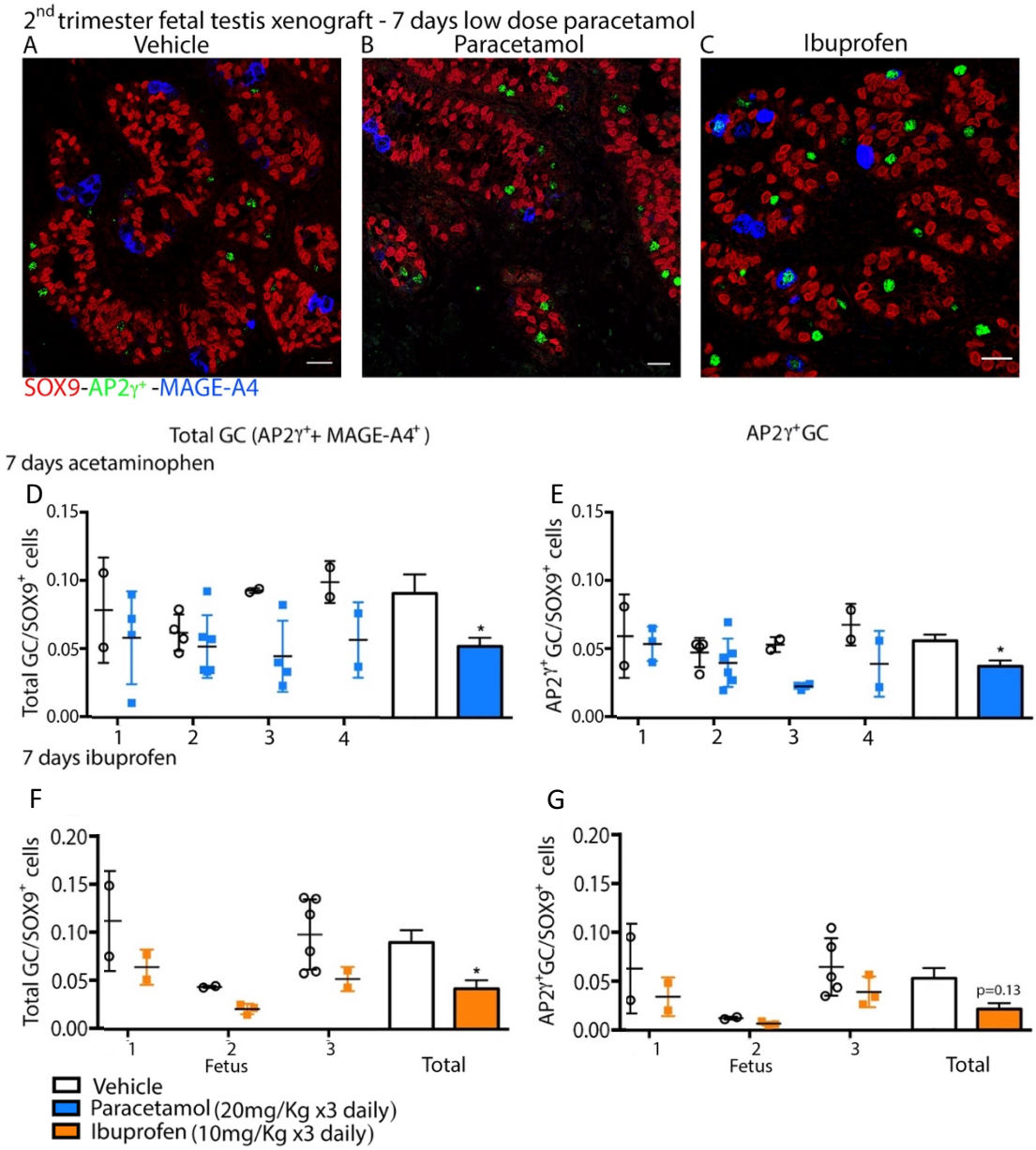


Figure 4.5. Effect of 7 days paracetamol or ibuprofen exposure of 2nd trimester fetal human testis tissue xenografts on germ cell number. Testis tissue pieces (~1mm^3) from 4 fetuses (7-day paracetamol) or 3 fetuses (ibuprofen), all within 14-17 gestational weeks, were xenografted into nude mice. Host mice were administered vehicle, paracetamol (20mg/kg x3 per day) or ibuprofen (10mg/kg x3 per day) for 7 days before xenograft recovery. Tissue was fixed and triple-immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC - blue) as depicted in panels **A**-**C**. Individual data points represent GC counts from a single tissue piece (replicates) in panel panels **D** and **E** for 7-days paracetamol exposure, panels **F** and **G** for ibuprofen exposure. Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA); *p<0.05, versus corresponding vehicle-exposed group. Corresponding MAGE-A4$^+$ GC counts and their % proliferation are shown in figures 4.6 and 4.7.

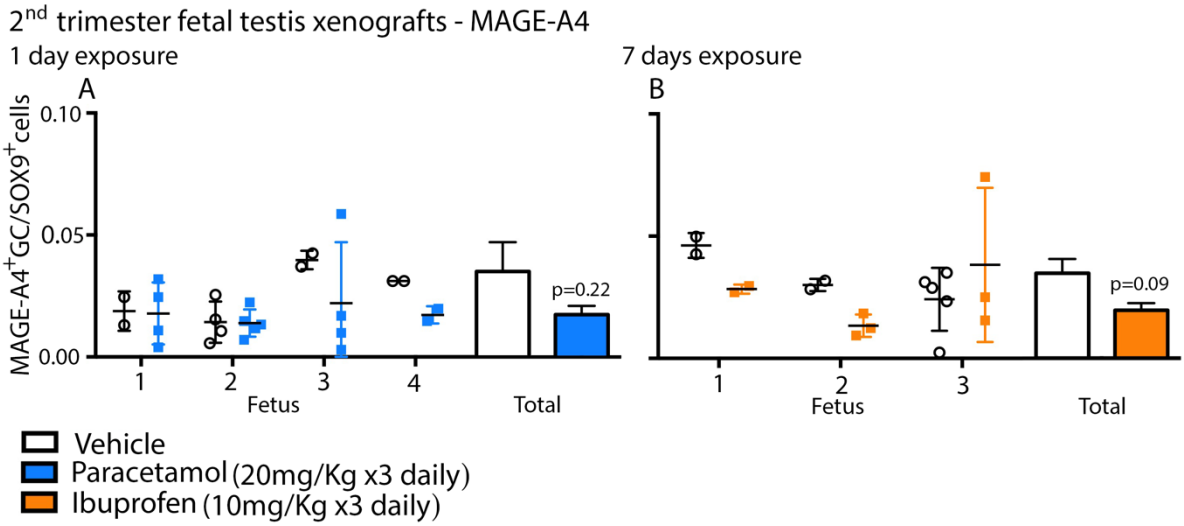


Figure 4.6. Effect of 7 days paracetamol or ibuprofen exposure of 2nd trimester fetal human testis tissue xenografts on MAGEA4+ germ cell number. Testis tissue pieces (~1mm^3) from 4 fetuses (7-day paracetamol) or 3 fetuses (ibuprofen) were xenografted into nude mice. Host mice were administered vehicle, paracetamol (20mg/kg x3 per day) or ibuprofen (10mg/kg x3 per day) for 7 days before xenograft recovery. Tissue was fixed and triple-immunostained for Ki67 (proliferating cells) and MAGE-A4 (subpopulations of GCs). MAGE-A4$^+$ GC counts for each tissue piece for each fetus for each treatment are shown by the individual symbols (replicates) in panels **A** and **B** for 7 days paracetamol and 7 days ibuprofen respectively. Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA).

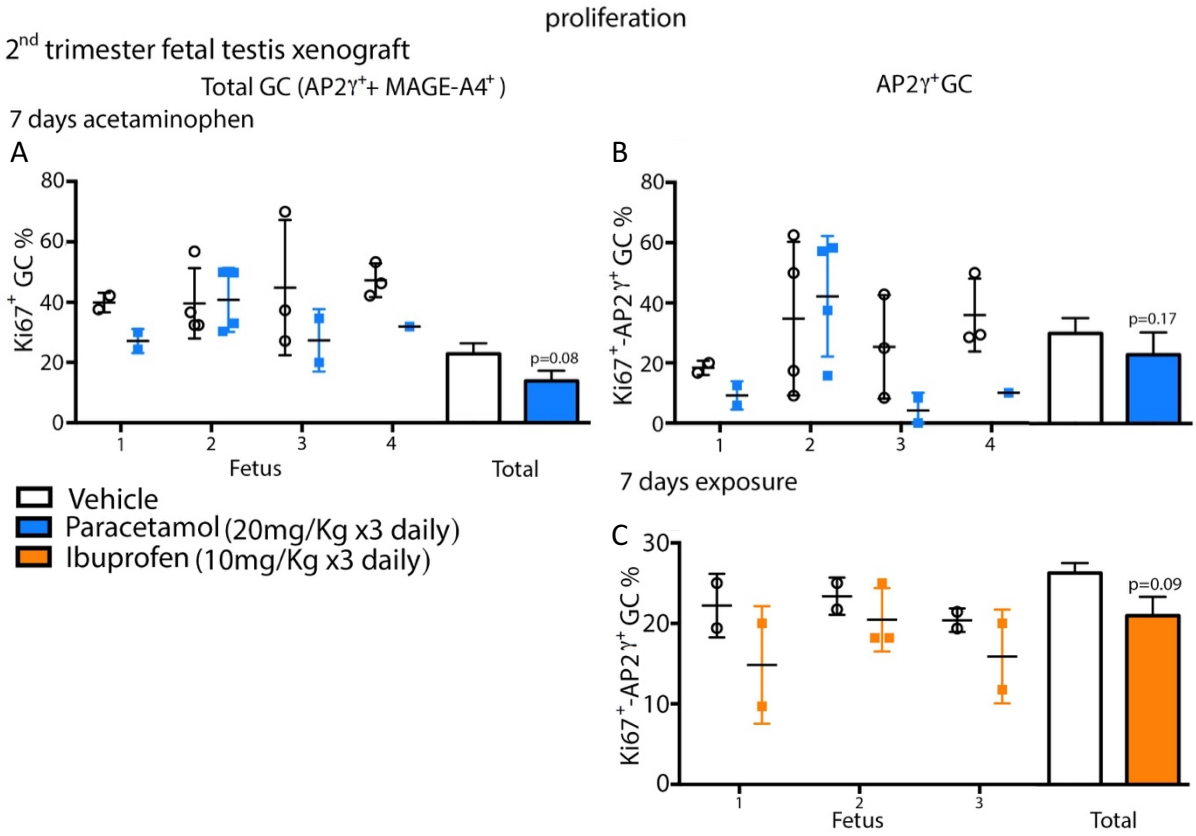


Figure 4.7. Effect of 7 days paracetamol or ibuprofen exposure of 2nd trimester fetal human testis tissue xenografts on germ cell proliferation. Testis tissue pieces (~1mm^3) from 4 fetuses (7-day paracetamol) or 3 fetuses (ibuprofen), all within 14-17 gestational weeks, were xenografted into nude mice. Host mice were administered vehicle, paracetamol (20mg/kg x3 per day) or ibuprofen (10mg/kg x3 per day) for 7 days before xenograft recovery. Tissue was fixed and triple-immunostained for AP2γ, MAGE-A4 and the proliferation marker Ki67. Quantification of the % of proliferative (Ki67$^+$) GC (AP2γ$^+$ or MAGE-A4$^+$) from paracetamol exposed samples (replicates) is shown on panel **A.** Quantification of the % of proliferative (Ki67$^+$) AP2γ$^+$ GC from paracetamol and ibuprofen are shown on panels **B** and **C.** Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA).

4.3.3 Effects of high dose paracetamol exposure for 7 days on GC in 2nd trimester human fetal testis xenografts

As described in chapter 3, rodent experiments used a higher paracetamol dose than the human relevant one, based on the calculations using body surface (Reagan-Shaw et al., 2008). In previous experiments with the xenograft model (van den Driesche et al., 2015), exposure for 7 days to a human relevant dose/regimen of paracetamol (20mg/kg/x3 daily) reduced seminal vesicle weight in the mice host, but no effect was found with a higher dose of paracetamol (350mg/kg/day) administered once per day for 7 days. I investigated whether GCs of the same 2nd trimester human testis xenografts were affected by this single high daily dose of paracetamol. However, similar to the previous results on seminal vesicle weight, it was found that this higher dose treatment regime did not have any effect on total GC number, nor on AP2γ^{+} or MAGE-A4^{+} GC subpopulations (corrected for Sertoli cell number) (Figure 4.8).

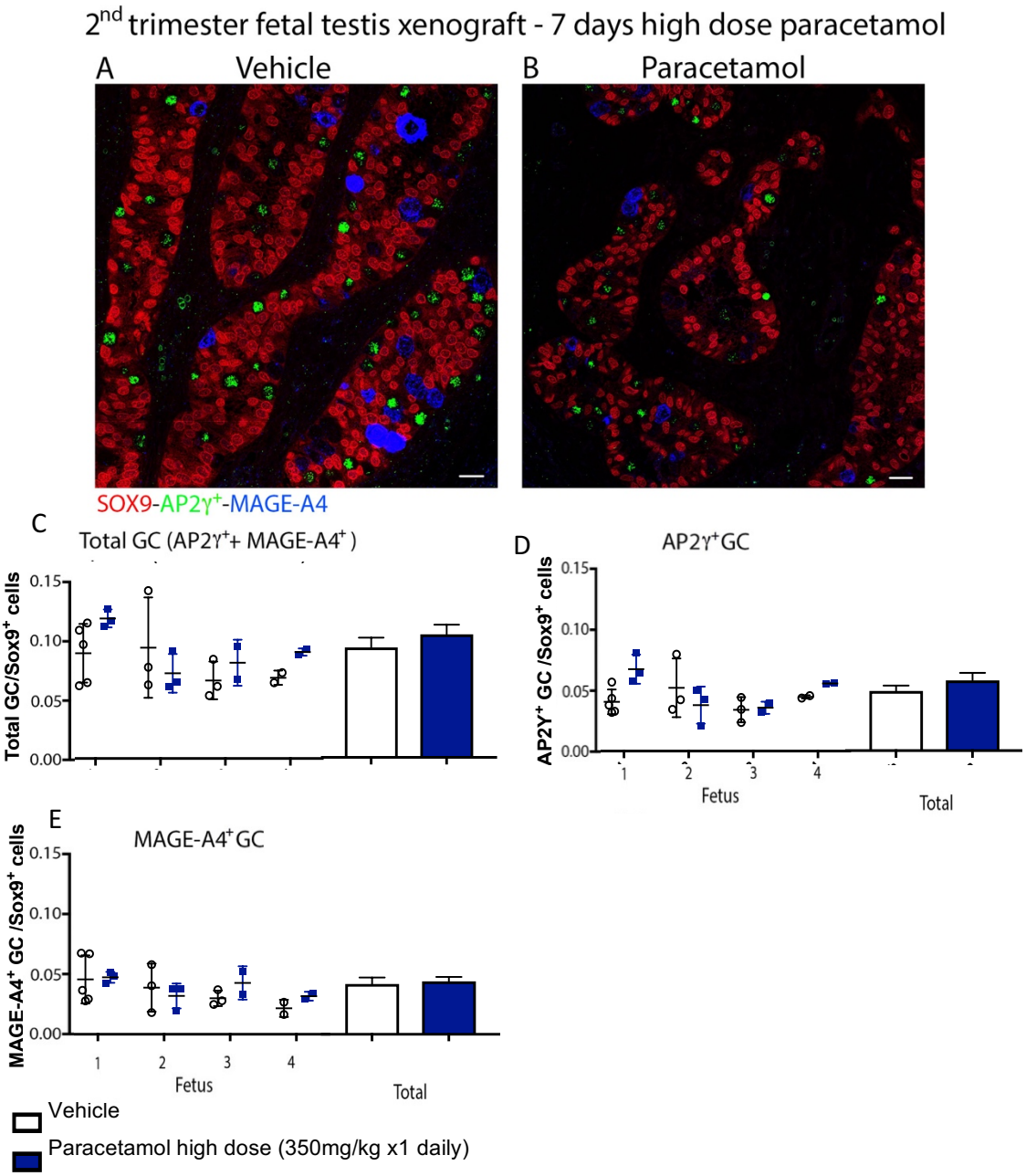


Figure 4.8. Effect of a single daily exposure of 2nd trimester fetal human testis tissue xenografts to high dose paracetamol for 7 days on germ cell number. Testis tissue pieces (~1mm^3) from 4 fetuses, all within 14-17 gestational weeks, were xenografted into nude mice. Host mice were administered vehicle or paracetamol (350mg/kg x1 daily) for 7 days before xenograft recovery. Tissue was fixed and triple-immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC - blue) as depicted in panels **A** and **B**. Individual data points represent GC counts from a single tissue piece (replicates) in panels **C-E**. Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA); *p<0.05, versus corresponding vehicle-exposed group.

4.3.3 Effects of 1 day (last day) paracetamol exposure on GC in 2nd trimester human fetal testis xenografts

The intake of analgesics by the general population, including pregnant woman, can be irregular and is more likely to be for short periods. For this reason, I investigated if short exposure to paracetamol (1 day) could also be detrimental to the fetal GC.. The analysis of GC using fluorescence immunohistochemistry for somatic cells and the different subpopulations of GCs showed that this regime did not cause any modification in total GC, AP2γ+ or MAGE-A4+ GC subpopulation numbers (corrected for Sertoli cell number), when compared with the vehicle-exposed group (Figure 4.9). Proliferation, studied by immunohistochemistry using Ki67 as a proliferation marker, was also unaffected by the treatment (Figure 4.9).

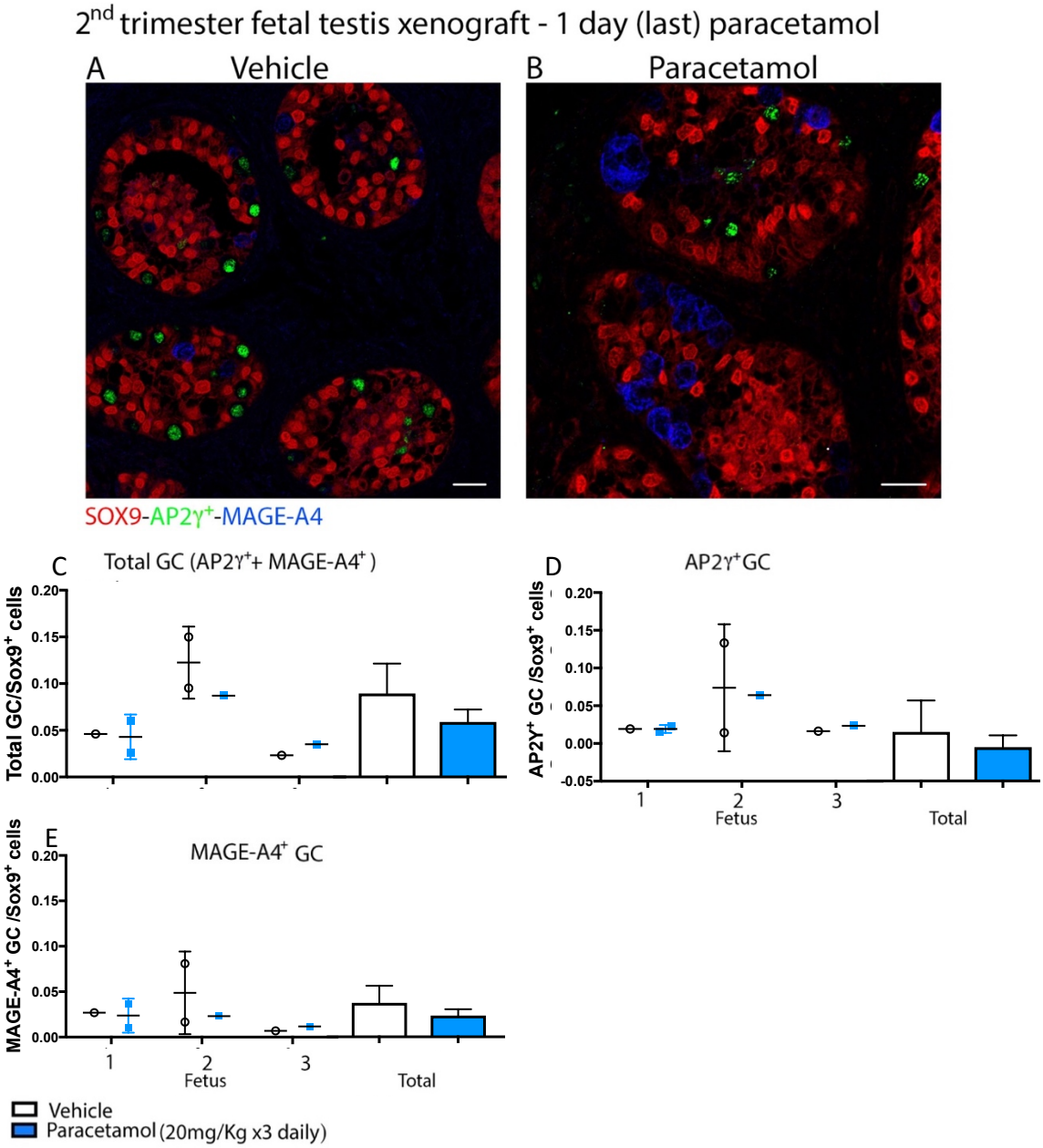


Figure 4.9. Effect of 1 day exposure (last) of 2nd trimester fetal human testis tissue xenografts to paracetamol on germ cell number. Testis tissue pieces (~1mm^3) from 4 fetuses, all within 14-17 gestational weeks, were xenografted into nude mice. Host mice were administered 1 day vehicle or paracetamol (20mg/kg x3 daily) before xenograft recovery. Tissue was fixed and triple-immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC - blue) as depicted in panels **A** and **B**. Individual data points represent GC counts from a single tissue piece (replicates) in panel panels **C-E**. Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA); *p<0.05, versus corresponding vehicle-exposed group.

4.3.3 Effects of 1 day (first day) paracetamol exposure on GC in 2nd trimester human fetal testis xenografts

In the previous experiment with 1 day paracetamol exposure, the samples were collected immediately after the paracetamol dose. If any delayed effects of the paracetamol exposure occurred, these may not have been evident by the sample day. To address this possibility, a similar experiment with paracetamol exposure for 1 day (20mg/kg x3) was performed, but in this case the mice were exposed to paracetamol the day after xenograft establishment (i.e. 7 days after xenografting), followed by vehicle exposure for the consequent 6 days. This 1 day paracetamol regime led to an overall significant reduction in total GC (22%; $p<0.05$) and AP2γ^+ GC number (17%; $p<0.05$) in xenografts (Figure 4.10), although these decreases were not as pronounced as found with the longer 7-day exposure (Figure 4.10). MAGEA4$^+$ GC subpopulation number was not significantly altered by 1-day paracetamol exposure using this exposure regime (Figure 4.10). GC proliferation was also studied using ki67 and did not show any significant reduction for either total GC or AP2γ^+ GC (Figure 4.10).

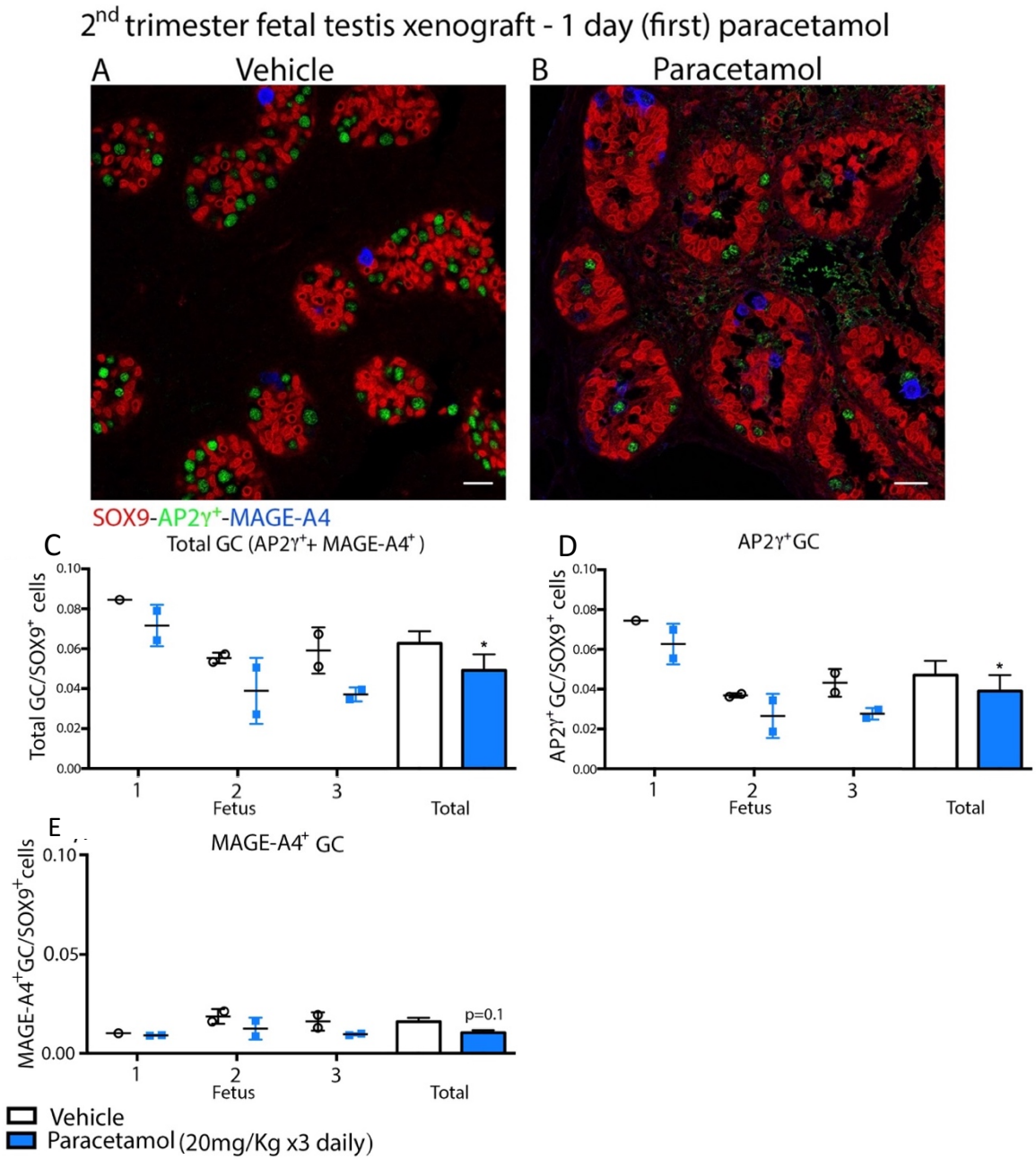


Figure 4.10. Effect of 1 day exposure (first) of 2nd trimester fetal human testis tissue xenografts to paracetamol on germ cell number. Testis tissue pieces (~1mm^3) from 4 fetuses, all within 14-17 gestational weeks, were xenografted into nude mice. Host mice were administered 1 day vehicle or paracetamol (20mg/kg x3 daily), followed by 6 days of vehicle doses before xenograft recovery. Tissue was fixed and triple-immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC - blue) as depicted in panels **A** and **B**. Individual data points represent GC counts from a single tissue piece (replicates) in panel panels **C-E**. Each n number is expressed as a different fetus (X-axis), together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA); *p<0.05, versus corresponding vehicle-exposed group.

4.3.3 Effect of analgesic exposure on prostaglandin E_2 production in 1st trimester human testis tissue in culture

The medium from the hanging drop cultures involving 7 day exposure to paracetamol or ibuprofen was collected each day, and the medium from all of the pieces from the same exposure group was pooled for analysis. PGE_2 content was measured on day 1 (no treatment), day 3 (48h exposure) and day 8 (7 days exposure) and whether it was modified by paracetamol or ibuprofen exposure of 1st trimester human testis tissue, using a commercial ELISA (section 2.7.). This revealed no difference in the PGE_2 content within the different treatment groups. However, there was an overall reduction of PGE_2 content in day 3 and day 8 samples, compared with day 1 cultures, which was comparable for all treatment groups (figure 4.11).

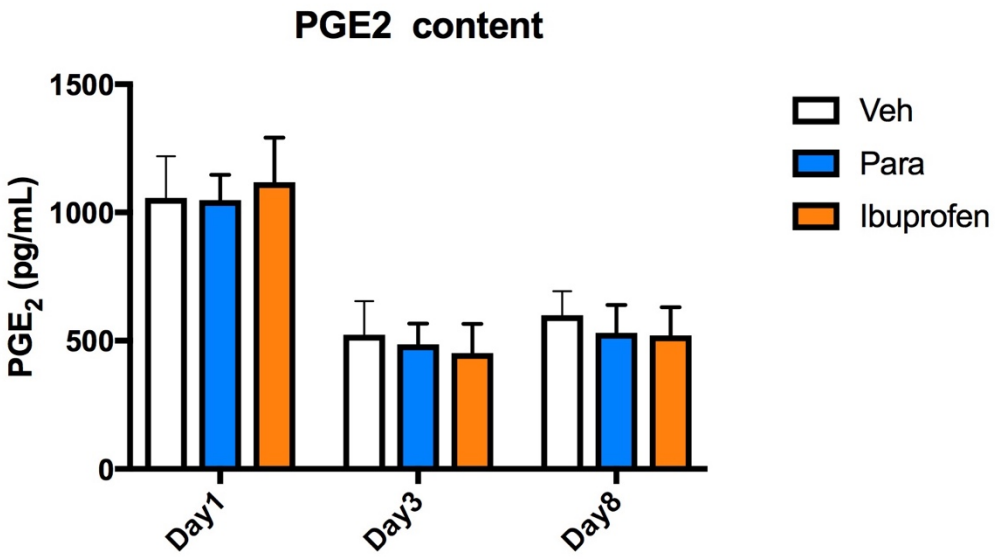


Figure 4.11. Effect of analgesic exposure of 1st trimester fetal human testis tissue for 7 days in hanging drop culture on medium PGE_2 content. Testis tissue pieces (~1mm^3) from 4 fetuses (8-11 gestational weeks) were cultured for 7 days with vehicle (White), paracetamol (10µM; blue) or ibuprofen (10µM; orange). PGE_2 content in the medium was measured on culture days 1, 3 and 8 and the averages of the 4 fetuses are expressed in pg/mL with the mean ± SEM for each treatment.

4.3 Discussion

The studies in this chapter investigated the consequences of paracetamol or ibuprofen exposure in two different human fetal gonad models: hanging drop culture for 1st trimester gonads (ovary and testis) or xenografting for 2nd trimester testes. In both model systems there was significant reduction in GC numbers after exposure to paracetamol or ibuprofen. Previous studies have focused on human fetal gonads exposed to paracetamol, aspirin or indomethacin (van den Driesche et al., 2015; Mazaud-Guittot et al., 2006). This is the first study involving ibuprofen exposure of human fetal gonads in model systems, which is the most relevant NSAID, together with aspirin, given the high percentage of women who report ibuprofen use during pregnancy (Werler et al., 2005). Different pieces of information are worth commenting on and are discussed below.

4.3.3 Human fetal gonad culture as a viable model

Human fetal gonads cultured in hanging drops have already been described previously (Jorgensen et al., 2012). This model offers a relatively easy culturing method in which the different cell types show good survival and the overall tissue shows maintenance of normal, healthy structure. The majority of the samples cultured with the different treatments in this project appeared healthy. However, it was observed that a degree of GC loss occurred as a consequence of the culture, when compared with pre-culture controls. This has been described previously by our lab. In general, GC are prone to undergo apoptosis if supporting conditions are not optimal, making them difficult to culture, including in hanging drops. For this reason, the cultures were supplemented with hCG, as previous studies in my lab had found that supplementing the medium with hCG helped to create an environment more similar to the pregnancy process and decreased the procedural loss of GC (van den Driesche et al., 2015).

4.3.3 Reduction in GC number after analgesic exposure of fetal human gonad tissue in hanging drop culture

1st trimester testis and ovary samples were cultured using the hanging drop system and exposed to paracetamol and ibuprofen for 7 days. In the testis, paracetamol exposure significantly reduced total GC number, but also the specific AP2γ^+ GC subpopulation. However, the population of MAGE-A4$^+$ GC in 1st trimester was too low to be counted. GC stop expressing AP2γ and start expressing MAGE-A4 during fetal gonad development, a process that starts during 1st trimester and slowly continues until the end of the 2nd trimester (Mitchell et al., 2008). This transition between AP2γ and MAGE-A4 is asynchronous and cells at different developmental stages are found in an individual seminiferous cord (Mitchell et al., 2010). As it was not possible to calculate the MAGE-A4$^+$ GC subpopulation number, the comparison of the trends among the different subpopulations in 1st trimester samples cannot be done. When focusing on ibuprofen, the treatment-induced reduction in GC was seen only for the AP2γ^+ GC with the decrease in total GC number being statistically non-significant. The lack of significance in the reduction of total GC number after ibuprofen exposure might be a consequence of the low number of fetuses studies. Possibly, with a higher N number, this result might be significant. When ovaries were exposed to the analgesics, they also showed similar GC changes to those found for the testis, with a significant decrease in AP2γ^+ GC.

The reduction in GC number seen in 1st trimester fetal gonads exposed to paracetamol or ibuprofen might be explained by the reduction seen in GC proliferation. Both analgesics significantly reduced the percentage of proliferative GC in testes and ovaries. How paracetamol and ibuprofen can affect GC proliferation has still to be addressed. Considering again the possibility that the effects of analgesics on fetal GC is through PGE_2, then previous studies have shown that PGE_2 is able to alter the proliferation of a wide variety of stem cells including fibroblasts, myocytes, T-cells, hepatocytes or skeletal muscle myoblasts (Sanchez & Moreno, 2002; Mendez & LaPointe, 2005; Krause et al., 2007; Kimura et al., 2001; Mo et al., 2015). One study

used mouse embryonic stem cells (mESc) as a model to study their proliferation after PGE_2 exposure (Yun et al., 2012). ESc are one of the closest cell types to GC and they share many of the characteristics and marker expression, such as the pluripotency factors, OCT4 and NANOG. Yun et al 2012 showed that PGE_2 increased proliferation (increased thymidine incorporation), but also increased cell number. Furthermore, they showed how increased PGE_2 modified expression of several common markers of proliferation, including some cyclins (Yun et al., 2012). If the contrary is also true, inhibition of the PGE_2 pathway, for example by paracetamol and ibuprofen, could result in reduced GC proliferation. This is further addressed in chapter 5.

The reduction in GC number seen as a consequence of paracetamol and ibuprofen exposure was more pronounced in ovaries than testes for 1st trimester samples. This difference between sexes raises the possibility of female GCs being more susceptible to analgesic exposure than male GCs. It is difficult to address the possible reasons for this apparent sex difference in susceptibility. Female GC could be more sensitive to the treatment for many different reasons, as they show different characteristics to male GC. For example, female fetal GC have only a short window during pregnancy in which to attain the final numbers of GC, whereas male GC proliferate for all of fetal life and beyond. This is perhaps the reason why male GC can “afford” to proliferate more slowly than females. Male and female GC proliferation is similar during the first weeks of gestation (Bendsen et al., 2006; Bendsen et al., 2003; Lutterodt et al., 2009; Mamsen et al., 2010; Mamsen et al., 2011). GC proliferation in both sexes is reduced by week 10 of gestation, although female GC proliferation remains at a higher rate than males (O'Shaughnessy et al., 2007; Fowler et al., 2009; Mamsen et al., 2011). This variation in GC proliferation among sexes could be one of the reasons for the different effect of paracetamol or ibuprofen exposure on fetal human GC number shown in this chapter. Other studies have shown how female GC are more affected by some specific treatments than male GC. For example, exposure of e13.5 mouse fetal testis and ovary cultures to the polycyclic aromatic hydrocarbon pollutant benzo [a] pyrene (BaP) revealed a significantly more pronounced increase in GC apoptosis

in females than in males (Lim et al., 2016). Another example, but in this case with a higher effect in the testis, is found after 4-octylphenol exposure, an endocrine disruptor that significantly reduced the GC number in human fetal testes cultured for 3 weeks, but had no effect on GC number in ovary culture (Bendsen et al., 2001). There is also the possibility that analgesics are affecting different pathways in the ovary and testis, translating into different levels of effect on GC numbers. For example, an increase in retinoic acid can advance GC meiosis entry (Le Bouffant et al., 2010; Baltus et al., 2006) and will therefore decrease the period of GC proliferation, which would be more important in ovary than testis. This would fit with the results previously described by this lab showing how paracetamol exposure increases Stra8 expression in the fetal rat ovary (Dean et al., 2016) and the results in section 3.3.1 showing increased expression of the retinoic acid synthesizer Aldha1 in fetal rat testes after in utero exposure to paracetamol or indomethacin.

4.3.3 Reduction in GC number in fetal testis xenografts after analgesic exposure

The xenograft model was used for 2nd trimester fetal testis samples and for 3 different treatments: 1 day paracetamol, 7 days paracetamol and 7 days ibuprofen. The three different treatments caused significant reductions in total GC. Taking into account only the 7 days exposures, reduction in total GC number was comparable with both paracetamol and ibuprofen. However, when focusing only on AP2γ^+ GC, only paracetamol exposure resulted in a statistically significant decrease in numbers. Ibuprofen exposed cultures showed a decrease in AP2γ^+ GC, although this was not statistically significant. This is probably because of the low number of fetuses in the ibuprofen experiments; it seems likely that if the sample number was higher, there would also have been a statistically significant reduction in AP2γ^+ GC after ibuprofen exposure of xenografts.

The experiments performed using a higher dose of paracetamol did not show any significant change in GC numbers in xenografts. It is important to note that the dose

regime of this system used only 1 dose of paracetamol per day instead of 3 doses per day for the lower dose, and it is the latter that more closely compares to normal human usage. Previous work by my lab also showed that this single high daily dose of paracetamol did not affect testosterone production in the host mice, whereas administration of the lower dose of paracetamol 3 times a day did significantly reduce testosterone production (van den Driesche et al., 2015). A possible explanation for this lack of consequences of high dose paracetamol exposure is that exposure is not prolonged enough. The fast metabolism of paracetamol may mean that the exposure window is too short after a single paracetamol dose to induce a reduction in GC number.

In 2nd trimester samples, the number of GC expressing MAGE-A4 was considerably increased compared with 1st trimester samples. None of the treatment regimes resulted in a significant decrease in MAGE-A4^{+} GC number, however, there was a general pattern of decrease in all the samples. Again, a higher N number in these experiments might potentially have shown a significant decrease in numbers of these pre-spermatogonia. Either way, the results suggest that this subpopulation of GC is less affected by the exposure to paracetamol or ibuprofen than are the AP2γ^{+} GC (gonocytes). This difference could be related to the notably lower proliferative rate of MAGE-A4^{+} GC compared to gonocytes (Mitchell et al., 2010). Therefore, if the mechanism of effect of analgesics is via inducing a lower proliferation rate, such an effect would be far more difficult to detect in GC with a low proliferation rate (i.e. pre-spermatogonia) than with a high rate (gonocytes).

Regarding GC proliferation in 2nd trimester human fetal testis xenografts, the percentages of proliferative GC were not significantly altered by the different treatment regimes. However, a similar rationale to the lack of decrease in MAGE-A4^{+} GC can be applied here. Proliferation rates in all analgesic-exposed samples showed a general pattern of decrease, although this failed to reach statistical significance in any of the individual cases. It is possible that the sample number is not high enough

for this experiment to show significance and that if the N number was increased sufficiently, a significant decrease in the GC proliferation rate would be found. Moreover, measuring proliferation rate at a specific time point is likely to be less sensitive than measuring actual GC number, as the latter is a compounded effect of reduced proliferation over the complete period of exposure/the experiment. Moreover, GC proliferative rates are decreased during the 2nd trimester as a result of a higher proportion of MAGE-A4^{+} GC, which are less proliferative. If there is a general decrease in GC proliferation, it is possibly more difficult to detect a decrease resulting from analgesic exposure.

The other interesting results to comment on are the comparison between the two different 1 day paracetamol regimes, but also to compare these with the 7 day paracetamol exposure group. Paracetamol use by pregnant woman is not regular and, in most women, the intake is likely to be for short periods (<24h). For this reason, I investigated whether shorter exposures (1 day) to paracetamol can also affect fetal GC number and/or proliferation using the 2nd trimester human fetal testis model. The difference between the two 1 day paracetamol regimes is interesting as it shows how the effect of paracetamol needs a longer time in order to have an effect on GC number and it is not an instant process. This is consistent with the effect on proliferation, because once GC number is reduced (due to reduced proliferation), even if this is a small effect and initially 'difficult to detect', this difference would become increasingly magnified with each subsequent round of proliferation, assuming no compensatory increase in the GC proliferation rate. This would provide a highly logical explanation for the difference in effect on GC number in xenografts of the 'late' versus 'early' 1-day exposure to paracetamol.

The decrease in GC number after just 1 day of paracetamol treatment raises a bigger concern about paracetamol exposure, because it is quite common for pregnant women to take paracetamol in this way. Nevertheless, my analysis also showed that there is a difference in scale of effect between 1 day and 7 days exposure to

paracetamol. While 1-day (early) exposure only reduced total GC number by 22% and gonocyte number by 17%, 7-day paracetamol exposure caused a 38% reduction in total GC number and a 58% reduction in AP2γ^+ GC number. The most logical explanation for this difference in the scale of effect is that, if the effect of analgesics is to reduce the GC proliferation rate, then clearly the longer the exposure the greater will be the scale of the effect. There might be more complex explanations, but in the absence of direct supporting evidence, those possible explanations cannot be further addressed. However, and regardless of the explanation of the more pronounced effect of longer exposures of paracetamol on reducing fetal GC number, the implication for use of paracetamol in pregnancy by women is clear. The shorter the use of paracetamol during pregnancy, the smaller any effects will be, which fits with present medical guidelines in the UK, but which should probably be reinforced into the general population by other sources.

4.3.3 Effect of analgesics on PGE_2 production by 1st trimester human gonad cultures

Paracetamol and ibuprofen can alter the PG pathway. Previous publications, including previous work by my lab have studied the effect of analgesics on PG production, especially PGE_2 and PDE_2 (Dean et al., 2016; Mazaud-Guittot et al., 2013; Kristensen et al., 2011). The results of these publications vary, with some of them reporting suppressive effects of analgesics on PG production by fetal gonads, and others reporting no significant effects of analgesic exposure. In my present studies, significant modification of the PGE_2 levels in the medium of 1st trimester testis cultures after exposure to paracetamol or ibuprofen were not found. The PGE_2 content measured was that released by the tissue/cells into the culture medium, which may reflect PGE_2 production, but will also be affected by metabolism within the tissue. However, PGs are rapidly metabolized, which will make the measurement of PGs levels in the media more complex (Ricciotti & FitzGerald, 2011; Schrey & Patel, 1995). Moreover, PGs are local factors and thus, measuring after diffusion from a tissue may not be very meaningful. Hence, it is not possible to be certain about the

reason for not finding an effect on PGE_2 concentration in the media of the hanging drop 1st trimester human fetal gonad culture, but it does not exclude the possibility for effects specifically on intracellular levels of PGE_2 and/or cell-specific effects of analgesics on PGE_2 specifically within GC or released from GC, which might have been masked in my whole fetal testis tissue cultures. This is addressed further in chapter 5.

4.3 Conclusion

In this chapter I have shown and discussed the possible effects of analgesics on human fetal gonads, with a specific focus on GC. The results showed how human relevant doses/exposure regimens of paracetamol and ibuprofen consistently caused reduced GC number in essentially all of the different model systems used: 1st trimester fetal testis and ovary exposed to 7 days paracetamol or ibuprofen and 2nd trimester testis exposed to either 1 or 7 days paracetamol or 7 days ibuprofen. Moreover, the analgesic-induced decrease in GC seemed to be more specific for the gonocyte subpopulation, although this could be a reflection of their higher proliferation rate compared with pre-spermatogonia (in males), which seemed to be rather less affected by the treatments. A possible explanation for the analgesic-induced decrease in GC number is that both paracetamol and ibuprofen are reducing GC proliferation. This was seen in 1st trimester samples, but the reduction was not significant in 2nd trimester fetal testes, although there was a consistent downward trend observed. This difference could be a reflection of the lower GC proliferation rate seen in 2nd trimester gonads as a consequence of the presence of more pre-spermatogonial cells, which are less proliferative (Mitchell et al., 2010). A decrease in fetal GC number can have different consequences, mainly regarding the potential fertility of the affected fetuses when they reach adult life. This is especially concerning in females because of their continuously reducing oocyte pool and their lack in repopulating GCs (Morita & Tilly, 1999) and hence, a reduction of fetal GCs in females will probably have more detrimental consequences. An example of this can be found in previous experiments by this lab using rats exposed in utero to

paracetamol or indomethacin, which showed how reduction in male fetal GC induced by these treatments was compensated for postnatally, with no consequences for fertility or adult testis weight. However, reduction in female fetal GC resulted in reduced adult ovarian weight and reduced fertility (Dean et al., 2016).

Overall, these results raise concern about analgesic use during pregnancy, but also suggest that restricting analgesic exposure as much as possible is likely to be safer in terms of the potential effects on fetal GC number in either sex.

Chapter 5: Analgesic exposure of NT2 cells as a human fetal germ cell model

5.1. Introduction

In chapters 3 and 4 I showed a number of possible consequences of analgesic exposure on the fetal gonad in rats and humans, involving reduction in GC number, but also effects on gonad/GC development. The human models were useful to study GC number, yet, due to the small availability of samples, it would be challenging to perform more advanced experimental studies on them. Thanks to the rat in vivo exposure and in vitro fetal gonad culture, I showed modifications to mechanisms by analgesics that could potentially explain these consequences, focused on SP proteins, but also on the epigenetic machinery. However, these rat models are not ideal to study the mechanisms altered by analgesics/prostaglandins in a deeper and more detailed way. The in vivo model has the disadvantage of not showing direct effects of analgesics on the gonads and the in vitro fetal gonad culture does not produce enough material for other types of studies, as well as having all of the limitations that go with any in vitro system. Moreover, many of the experiments performed in these models, such as qPCR, are not necessarily GC specific, but reflect the expression changes of the whole gonad. For that reason, a more tractable system, the Ntera-2 (NT2) cellular system was used. NT2 is a human embryonal carcinoma (germ cell tumour) cell line. Despite the fact that NT2 cells derive from adult cells, they show many characteristics of fetal GC, including the expression of some fetal GC pluripotency markers, such as AP2γ (Hoei-Hansen et al., 2004). The widely accepted mechanism to explain the origin of testicular GC cancer (TGCC) is the arrested differentiation of fetal GC, explaining the similarities between fetal GC and TGCC cells (Mitchell et al., 2014), including NT2 cells.

Previous experiments in this thesis (rat fetal gonad culture and NT2 cell culture, above) revealed that exposure to PGE_2 receptor antagonists produced comparable results to paracetamol or ibuprofen exposure. However, this is not enough proof to confirm that both analgesics are acting through the same pathways as PGE_2. In order to clarify this, new experiments were performed using EP2 and EP4 agonists.

Throughout this chapter, I will show and discuss the experiments performed exposing NT2 cells to paracetamol, ibuprofen and EP2+EP4 antagonists. The experiments of this chapter were based on the results seen with the rat and human models in the previous chapters and on different previous studies showing some of the effects that PGE_2 can have in different cell types, including cell number (and proliferation), pluripotency state and epigenetic regulators (Yun et al., 2009; Yun et al., 2012; Xia et al., 2012; Arosh et al., 2015), explained in section 1.5.3. To do so, NT2 number was analysed, as well as their proliferation phase state and gene expression profile after the different treatments. Following the similar results seen between EP2+EP4 antagonists and analgesics on fetal rat gonad cultures, another main focus was to study if the PGE_2 receptor antagonists could also mimic the effects of paracetamol and ibuprofen on NT2 cultures. Furthermore, two other different exposures were used as a treatment for the NT2 cells. On the one hand, EP2+EP4 agonists were used to see if they could prevent or modify some of the phenotypes seen after paracetamol exposure. On the other hand, specific modulators of EZH2 and the histone demethylase JMDJ3 were used in order to modify exclusively the activity of the PRC2 complex and to find possible similarities with analgesic exposure.

5.2. Material and Methods

NT2 cell treatments involved supplementing media with either vehicle (DMSO), 10 or 50µM paracetamol, 10µM ibuprofen or 10µM L-161,982 (EP2 antagonist) + 10µM PF04418948 (EP4 antagonist). Further analyses were done using EP2+EP4 agonists to see if the phenotypes seen with paracetamol could be rescued. For the rescue analysis, NT2 cells (excluding vehicle) were first exposed to 10µM Butaprost (EP2 agonist) and 10nM CAY10598 (EP4 agonist) for 4h before the medium was changed to the subsequent treatment: 10µM Butaprost + 10nM CAY10598, 10µM paracetamol or the combination of paracetamol + EP2+EP4 agonists. Furthermore, NT2 cells were exposed to 0.3µM GSK-126 (EZH2 inhibitor) and 4µM GSK-J4 (JMJD3 inhibitor) inhibitors to modify the PRC2 complex and see possible similarities with analgesic exposure. The analgesic doses used were selected because they reflect the

lower end of the range of serum concentrations in humans after normal therapeutic analgesic exposure (Mazaud-Guittot et al., 2013; Kristensen et al., 2012; Janssen & Venema, 1985). The EP2+EP4 agonist/antagonist doses were obtained from previous studies (Coskun et al., 2013; Fujimori et al., 2012; af Forselles et al., 2011). The doses of EZH2 and JMJD3 used were also taken from previous studies (Xie et al., 2016; Hashizume et al., 2014).

$2x10^5$ cells were cultured in 6-well plates and all cultures were exposed for a total time of 48 hours. In a single experiment, every treatment was run in triplicate and each experiment was performed three or four times (n=3-4). After treatment, cells were either collected for gene expression analyses with Trizol or disaggregated with TRypLE for calculating the cell number and cell cycle stage or for protein analysis by ELISA. The total number of cells at the end of the experiment was determined using a NucleoCounter, which allows a rapid cell count (section 2.6.5). Flow cytometry was used to study the cell cycle stage of the cells and also to determine the percentage of cell death at the end of the experiment. RNA from cultured cells exposed to the different treatments was extracted for qPCR analyses. RNA expression was analysed using Taqman technology to study a wide variety of genes including pluripotency markers and epigenetic regulators as described in section 2.8.6. The histone modifications H3K27me3 was also analysed by Elisa (section 2.7).

5.3. Results

5.3.1. Effect of paracetamol, ibuprofen and EP2+EP4 antagonists on NT2 cell number

$2x10^5$ NT2 cells were exposed for 48h to different doses of paracetamol (10-50μM), ibuprofen (10μM) or EP2+EP4 antagonists (10μM each). All these different exposures significantly reduced the number of NT2 cells after 48h exposure, with a dose response effect for paracetamol (Figure 5.1).

5.3.2. Exposure of EP2+EP4 agonists on NT2 cells

NT2 cells were exposed to either EP2+EP4 agonists, paracetamol or EP2+EP4 agonists in conjunction to paracetamol for 48h before being counted. In this way, if paracetamol is acting through the PGE_2 pathway, EP2+EP4 agonists should be able to protect the NT2 cells from the paracetamol effect. NT2 cells were exposed first to EP2+EP4 agonists for 4h before media was supplemented with paracetamol. EP2+EP4 agonist exposure did not itself produce any modification of NT2 cell number, while paracetamol exposure alone significantly reduced it. The combination of paracetamol and PGE_2 agonists resulted in NT2 cell numbers similar to vehicle and PGE_2 agonists alone and a significantly higher cell number than after culture with paracetamol alone (Figure 5.1D).

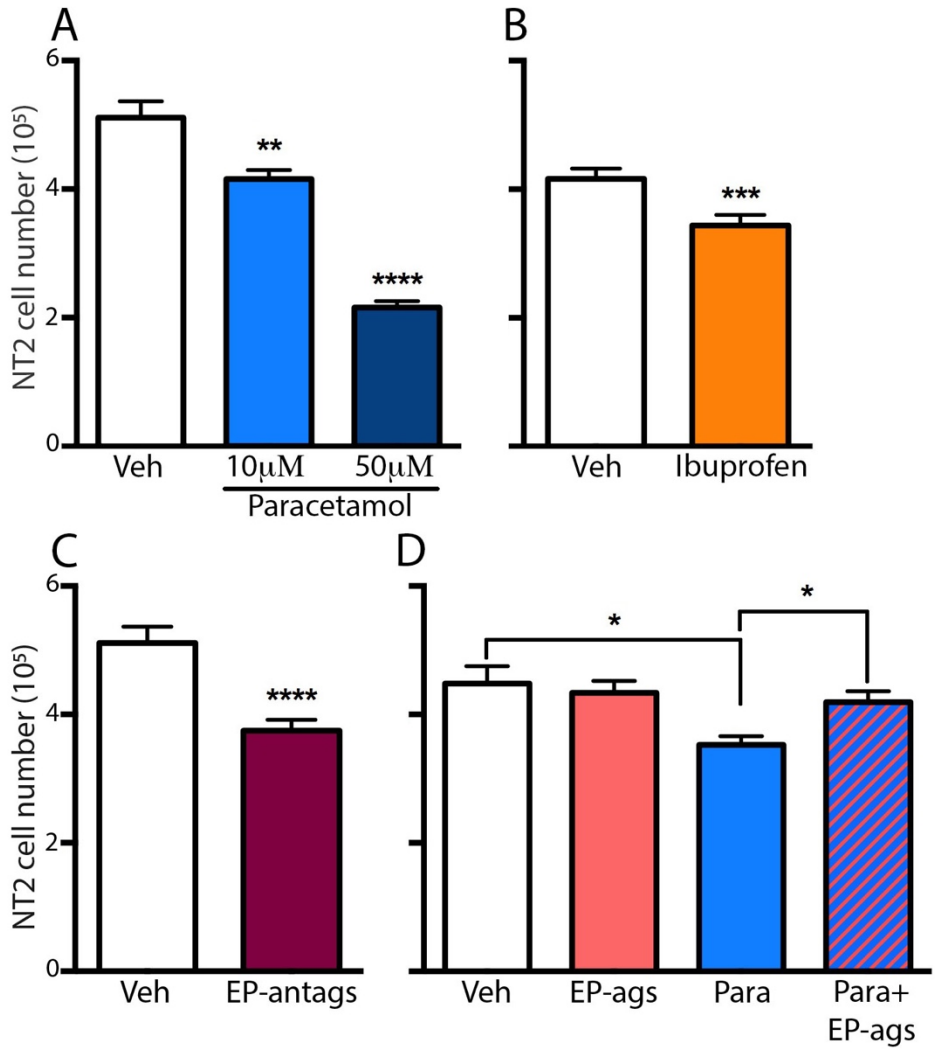


Figure 5.1. Effect of exposure of NT2 cells to analgesics or prostaglandin E2-receptor modulators on cell number. NT2 cells ($2x10^5$; n=3-4) were cultured for 48h in medium supplemented with either vehicle (Veh), paracetamol (10-50μM), ibuprofen (10μM), EP2+EP4 antagonists [EP-antags: 10μM L-161,982 (EP2 antagonist) + 10μM PF04418948 (EP4 antagonist)], or with EP2+EP4 agonists [EP-ags: 10μM Butaprost (EP2 agonist) + 10nM CAY10598 (EP4 agonist)] or the combination of paracetamol + EP2/EP4 agonists (Para+EP-ags). The mean ± SEM NT2 cell number for each treatment is shown in panels **A** – **D.** Panel **D** shows that co-culture of NT2 cells with PGE_2 agonists blocks the negative effect of paracetamol exposure on cell number. Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus corresponding vehicle-exposed group. Panel **D** analysis was followed by a comparison between Para and Para+EP-ags columns. Corresponding data for NT2 cell death as a consequence of the different treatments is shown in Figure 5.2.

5.3.3. Effect of paracetamol, ibuprofen and EP2+EP4 antagonists on NT2 cell death

The decrease in cell number seen as a consequence of the different treatments on the NT2 cells might be derived from an increase in cell death. However, the study of cell death can be challenging. Dead cells in the media are difficult to quantify, as they might be degraded and apoptosis enzymes metabolized. Hence, percentage of dead NT2 cells was calculated at the end of the experiment with the different treatments using flow cytometry. Propidium iodide (PI), which has the capacity of entering dead cells, was added to the disaggregated cells and the percentage of dead cells at the time of the end of the experiments was calculated and compared between treatments. Cell death was significantly increased by both doses of paracetamol and EP2+EP4 antagonists after 48h exposure (Figure 5.2). However, this increase in cell death was quite minor with low dose paracetamol and EP2+EP4 antagonists. The higher dose of paracetamol did produce a considerable increased in cell death.

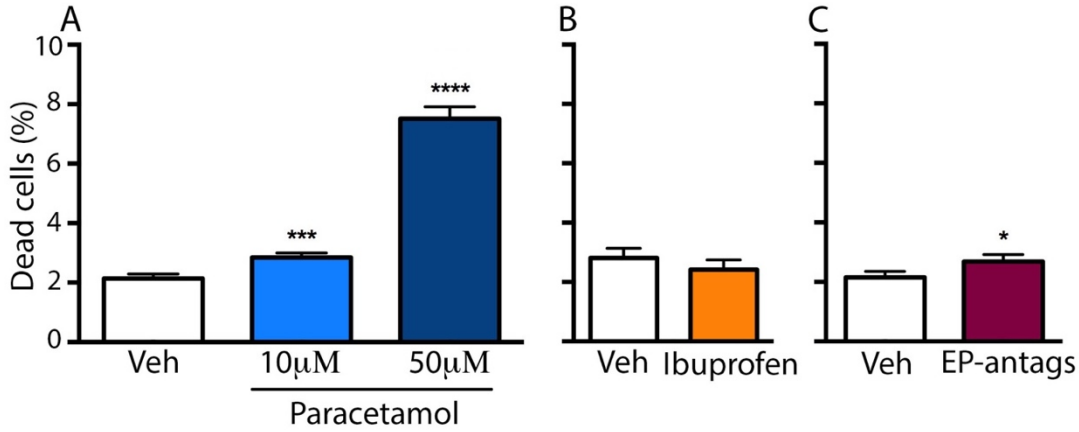


Figure 5.2. Effect of exposure of NT2 cells to analgesics or prostaglandin E2-receptor modulators on cell death. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with either vehicle (Veh), paracetamol (10-50μM), ibuprofen (10μM) or EP2+EP4 antagonists [EP-antags: 10μM L-161,982 (EP2 antagonist) + 10μM PF04418948 (EP4 antagonist)]. The % of NT2 dead cells at the end of the experiment for each treatment is shown in panels **A** – **C.** Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, ***p<0.001, ****p<0.0001 versus corresponding vehicle-exposed group.

5.3.4. Effect of paracetamol, ibuprofen and EP2+EP4 antagonists on NT2 cell cycle

Paracetamol and ibuprofen exposure decreased GC number in the human fetal gonad models (1st trimester fetal gonad culture, section 4.3.2 and 4.3.3; 2nd trimester xenografts, section 4.3.4 and 4.3.7) and in NT2 cell cultures (above). The results in 1st trimester human fetal gonad culture also showed how this reduction in GC number was possibly due to a reduction in GC proliferation (Section 4.3.2). Hence, it was studied if the reduction in cell number seen in NT2 cell cultures after exposure to paracetamol, ibuprofen and EP2+EP4 antagonists was also related to a reduction in proliferation. Cell proliferation was studied using Hoechst staining by flow cytometry. Hoechst is a cell-permeant nuclear counterstain able to bind to dsDNA, emitting blue fluorescence. Cells in G2/Mitosis (G2/M) have the double amount of DNA than a cell in G0/G1, and cells in synthesis phase have an intermediate between G0/G1 and G2/M. Therefore, Hoechst staining allows distinction of the cells according to their cell stage depending on their DNA content. With this method, the percentage of cells in each cell cycle stage (G0/G1, Synthesis or G2/M) was calculated using flow cytometry and the BD Facs software (section 2.6.7).

The results of these analysis showed differences between vehicle and treatment-exposed samples, but also within treatments. NT2 cells treated for 48h with low or high doses of paracetamol showed a decreased percentage of cells in G0/G1, shifting to synthesis (S) and especially to G2/mitosis (G2/M), when compared to vehicle (Figure 5.3A). In contrast, Ibuprofen exposure of NT2 cells did not produce any significant pattern or difference in any of the cell stages, compared to vehicle exposed cells (Figure 5.3B). When cells were exposed to EP2+EP4 antagonists for 48h, the percentage of cells in G2/M compared with vehicle was reduced, although this was not reflected in a variation of percentage of cells in other cell cycle stages (Figure 5.3C).

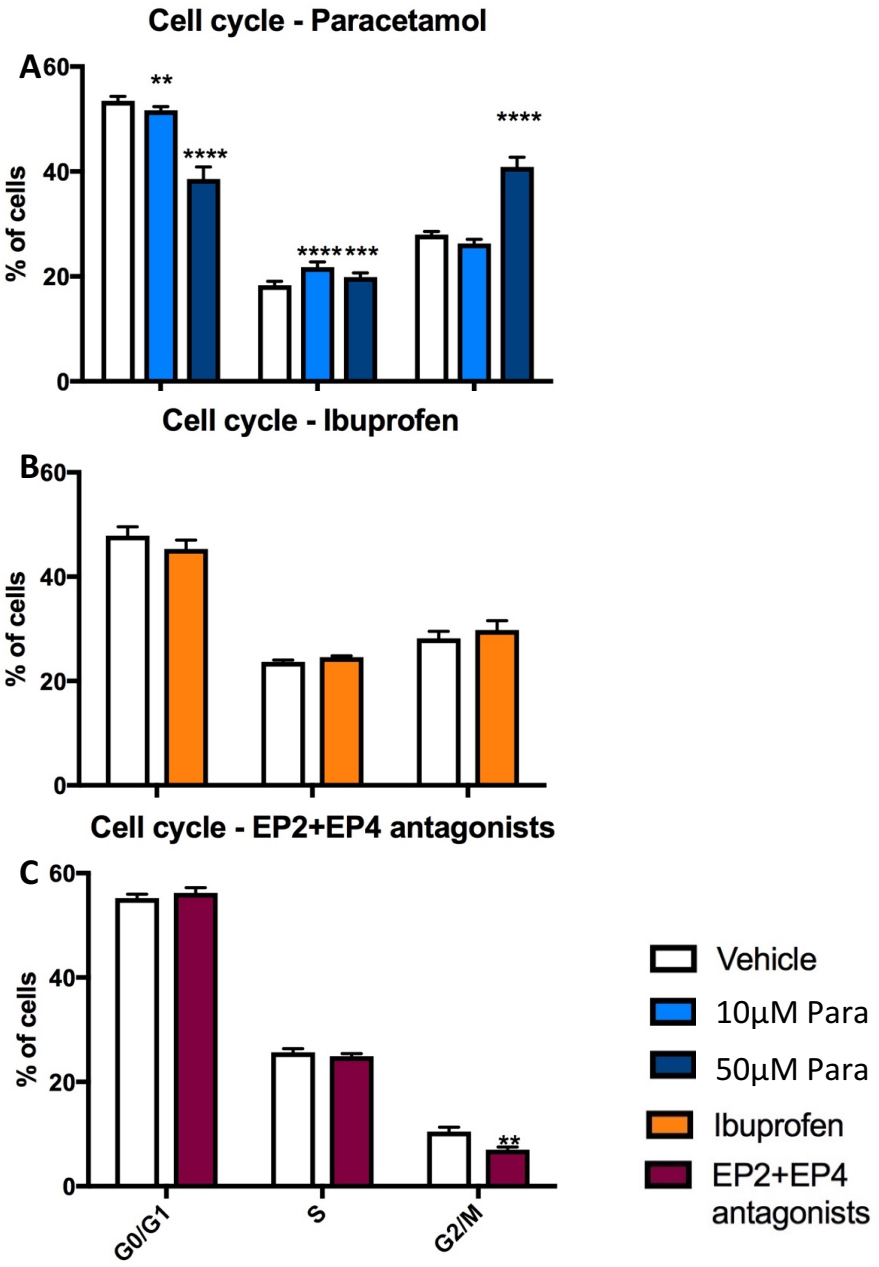


Figure 5.3. Cell cycle stage of NT2 cells exposed to paracetamol, ibuprofen or EP2+EP4 antagonists. NT2 cells ($2x10^5$; n=3) were cultured for 48h in medium supplemented with either vehicle (Veh), paracetamol (10-50µM; **A**), ibuprofen (10µM; **B**) or EP2+EP4 antagonists [EP-antagonists: 10µM L-161,982 (EP2 antagonist) + 10µM PF04418948 (EP4 antagonist); **C**]. Each panel represents the % of cells in the different cell cycle stages with the different treatments. Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus corresponding vehicle-exposed group.

5.3.4.1. Effect of paracetamol and EP2+EP4 antagonists on NT2 cell cycle (qPCR)

In light of the changes in the cell cycle stages depending on the different treatments of NT2 cells, I decided to perform a more detailed cell cycle analysis of NT2 cells exposed to paracetamol and EP2+EP4 antagonists. To do so, the expression of a wide range of genes associated with specific stages of the cell cycle (Grant et al., 2013) was quantified. I analyzed the expression of E2F transcription factor 2 (*E2F2*), which binds to the retinoblastoma protein in a cell-cycle dependent manner and Cyclin E1 (*CCNE1),* important in the formation of a complex with cyclin dependent kinase 2 (CDK2), for G1/S. For synthesis phase, the expression of histone cluster 1 H4 family member 4 (*HIST1H4B)* and ribonucleotide reductase regulatory subunit M2 (*RRM2),* which catalyzes the formation of deoxyribonucleotides from ribonucleotides, was determined. Cyclin A2 (*CCNA2*), in charge of activating CDK2*,* was used as a G2 marker and Cyclin B1 (*CCNB1)* and the mitotic checkpoint seronine/threonine kinase *BUB1,* as G2/M markers. Lastly, cohesion complex component *RAD21* and pituitary tumour-transforming 1 (*PTTG1)* was studied to analyze the M/G1 phase.

Similar to the cell cycle analysis by flow cytometry, the results showed differences between paracetamol and EP2+EP4 antagonists after 48h exposure of NT2 cells. Both doses of paracetamol reduced the expression of *E2F2* (borderline significance for high dose), a gene expressed in G1, although it did not modify the expression of another G1 marker, *CCNE1* (Figure 5.4). On the study of the synthesis phase, the expression of *HIST1H4B* was increased for the higher dose of paracetamol, but no difference for *RRM2* was found for either dose of paracetamol (Figure 5.4). In the case of *CCNB1* and *BUB1*, genes used for the study of the transition between G2 and mitosis, their expression was significantly increased by paracetamol exposure, except BUB1 for the lower dose of paracetamol (p=0.06). *RAD21* and *PPTG1*, both used as markers of transition between mitosis and G1 showed significantly decreased expression after NT2 cell exposure to either paracetamol dose (Figure 5.4).

In the case of NT2 cell exposure to EP2+EP4 antagonists, no significant modification of expression was found for any of the genes studied, except for the expression of *HIST1H4B* (synthesis marker), which was increased (Figure 5.5).

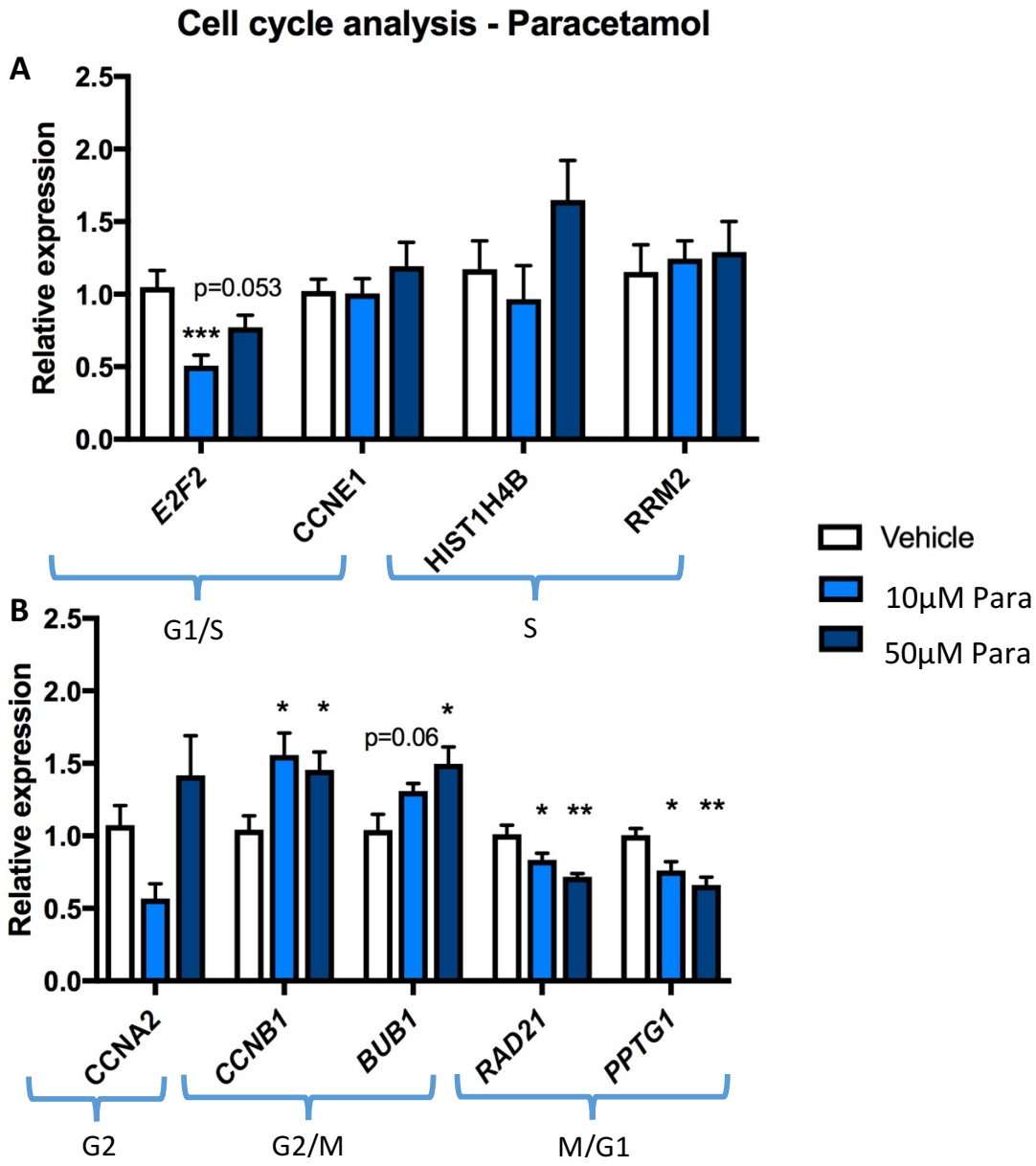


Figure 5.4. Effect of exposure of NT2 cells to paracetamol on mRNA expression of cell cycle markers. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with vehicle or paracetamol (10µM - LD para; 50µM – HD Para). Results show mRNA expression relative to the vehicle complementary DNA (open bars) after exposure of NT2 cells to the different doses of paracetamol. (**A**) G1/Synthesis markers E2F2 and CCNE1 and the Synthesis phase markers HIST1H4B and RRM2. (**B**). G2 marker CCNA2, G2/M markers CCNB1 and BUB1 and M/G1 markers RAD21 and PPTG1. Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, **p<0.01, ***p<0.001, in comparison with corresponding vehicle or vehicle complementary DNA.

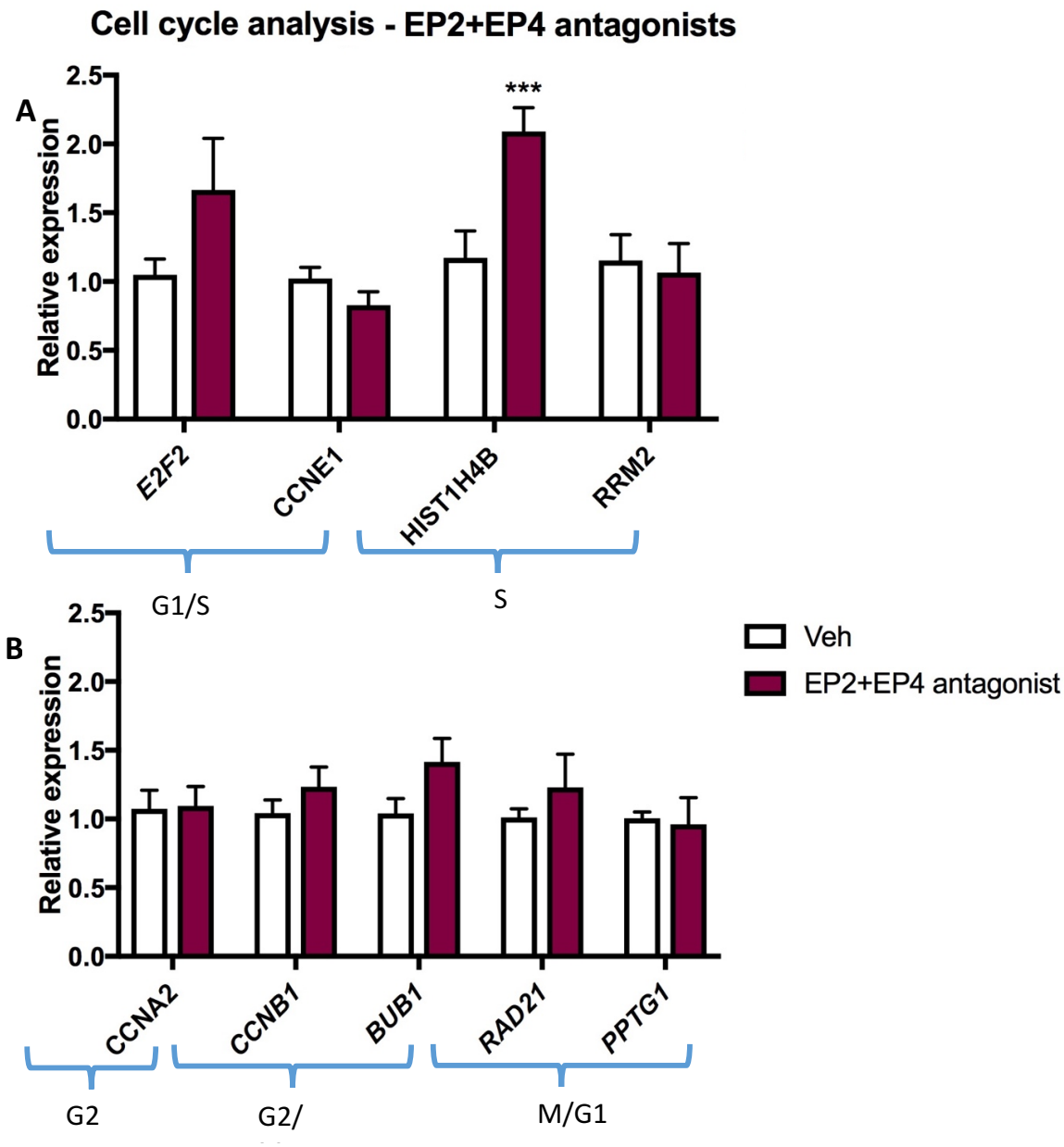


Figure 5.5. Effect of exposure of NT2 cells to prostaglandin E_2 antagonists on mRNA expression of cell cycle markers. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with vehicle or EP2+EP4 antagonists [EP-antags (10µM L-161,982; EP2 antagonist + 10µM PF04418948; EP4 antagonist)]. Results show mRNA expression relative to the vehicle complementary DNA (open bars) after exposure of NT2 cells to EP2+EP4 antagonists. (**A**) G1/Synthesis markers E2F2 and CCNE1 and the Synthesis phase markers HIST1H4B and RRM2. (**B**). G2 marker CCNA2, G2/M markers CCNB1 and BUB1 and M/G1 markers RAD21 and PPTG1. Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; ***p<0.001, in comparison with corresponding vehicle or vehicle complementary DNA.

5.3.5. Effect of paracetamol, ibuprofen and EP2+EP4 antagonists on NT2 pluripotency markers gene expression

Previous studies by our lab showed that analgesic exposure of fetal rat testes during gestation resulted in premature loss of expression of the gonocyte marker Oct4 (Dean et al., 2016). It was therefore investigated if NT2 cells exposed to paracetamol, ibuprofen or EP2+EP4 antagonists showed altered expression of established GC differentiation markers, such as OCT4, AP2γ or NANOG (Rajpert-De Meyts, 2006). Paracetamol exposure significantly reduced NT2 expression of the pluripotency markers *OCT4*, *AP2*γ and *NANOG* (Figure 5.6A). Ibuprofen led to a significant reduction in *OCT4* expression, but did not modify expression of the rest of the pluripotency markers (Figure 5.6B). Similar to paracetamol, NT2 cells cultured with EP2+EP4 antagonists significantly reduced expression of *OCT4*, *AP2*γ and *NANOG* (Figure 5.6C).

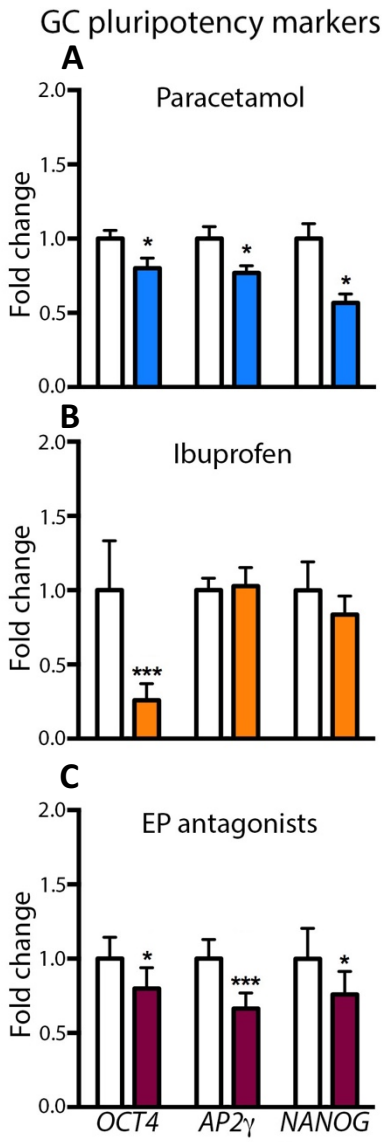


Figure 5.6. Effect of exposure of NT2 cells to analgesics or prostaglandin E_2 receptor antagonists on mRNA expression of GC pluripotency markers. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with vehicle (Veh), paracetamol (Acet; 10-50µM), ibuprofen (Ibu; 10µM) or EP2+EP4 antagonists [EP-antags (10µM L-161,982; EP2 antagonist + 10µM PF04418948; EP4 antagonist)]. Results show mRNA expression relative to the vehicle complementary DNA (open bars) for the GC pluripotency markers *OCT4*, *AP2γ* and *NANOG* (**A- C and E**) after exposure of NT2 cells to paracetamol (blue), ibuprofen (orange) or EP2+EP4 antagonists (maroon) (n=3). Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, **p<0.01, ***p<0.001, in comparison with corresponding vehicle or vehicle complementary DNA.

5.3.6. Effect of paracetamol, ibuprofen and EP2+EP4 antagonists on expression of epigenetic regulatory genes in NT2 cells

Previous studies by this lab showed inter-generational consequences as a result of analgesic exposure of pregnant rats (Dean et al., 2016). There is other published evidence that PGE_2 may regulate the epigenetic machinery in various tissues/cells (Xia et al., 2012; Venza et al., 2012; Arosh et al., 2015). Moreover, as shown in chapter 3, exposure to paracetamol, ibuprofen or EP2+EP4 antagonists seem to exert effects on the expression of epigenetic regulatory genes in rat fetal gonads when exposed in utero or when cultured to paracetamol (Section 3.3.4 and 3.3.7). Hence, I studied if similar consequences of analgesics on epigenetic regulators were also applicable for NT2 cells when exposed to paracetamol, ibuprofen or EP2+EP4 antagonists. Similar to the rat models, DNA and histone methylation were the main focus. Thus, the expression of the DNA methyltransferases *DNMT3A* and *DNMT3B* was studied, but also *TET1* expression, which plays a role in DNA and histone methylation and *EZH2* expression, which is the enzymatic component of the polycomb repressive group 2 (PRC2) complex, responsible for H3K27me3 histone methylation, a repression marker.

DNMT3A expression was unaffected in NT2 cells by 48h exposure to paracetamol, ibuprofen or EP2+EP4 antagonists, whereas *DNMT3B* expression was reduced only after culture with paracetamol or EP2+EP4 antagonists (Figure 5.7A-C). Conversely, expression of *TET1* was increased when NT2 cells were cultured for 48h with paracetamol, ibuprofen or EP2+EP4 antagonists, while *EZH2* expression was only increased significantly by EP2+EP4 antagonists (Figure 5.7A-C).

Increased expression of *TET1* could result in increased recruitment of the PRC2 complex, confirmed by the increased expression of *EZH2*. The PRC2 complex is in charge of trimethylating lysine 27 of histone 3 (H3K27me3). The increase in *TET1* and/or *EZH2* seen in NT2 cells after exposure to paracetamol, ibuprofen or EP2+EP4 antagonists, could result in increased H3K27me3. Therefore, total H3K27me3

present in NT2 cells after 48h culture with the different treatments was determined by ELISA (section2.7). H3K27me3 was significantly increased, relative to the total amount of protein, after culture of NT2 cells with paracetamol, ibuprofen or the EP2+EP4 antagonists (Figure 5.7D).

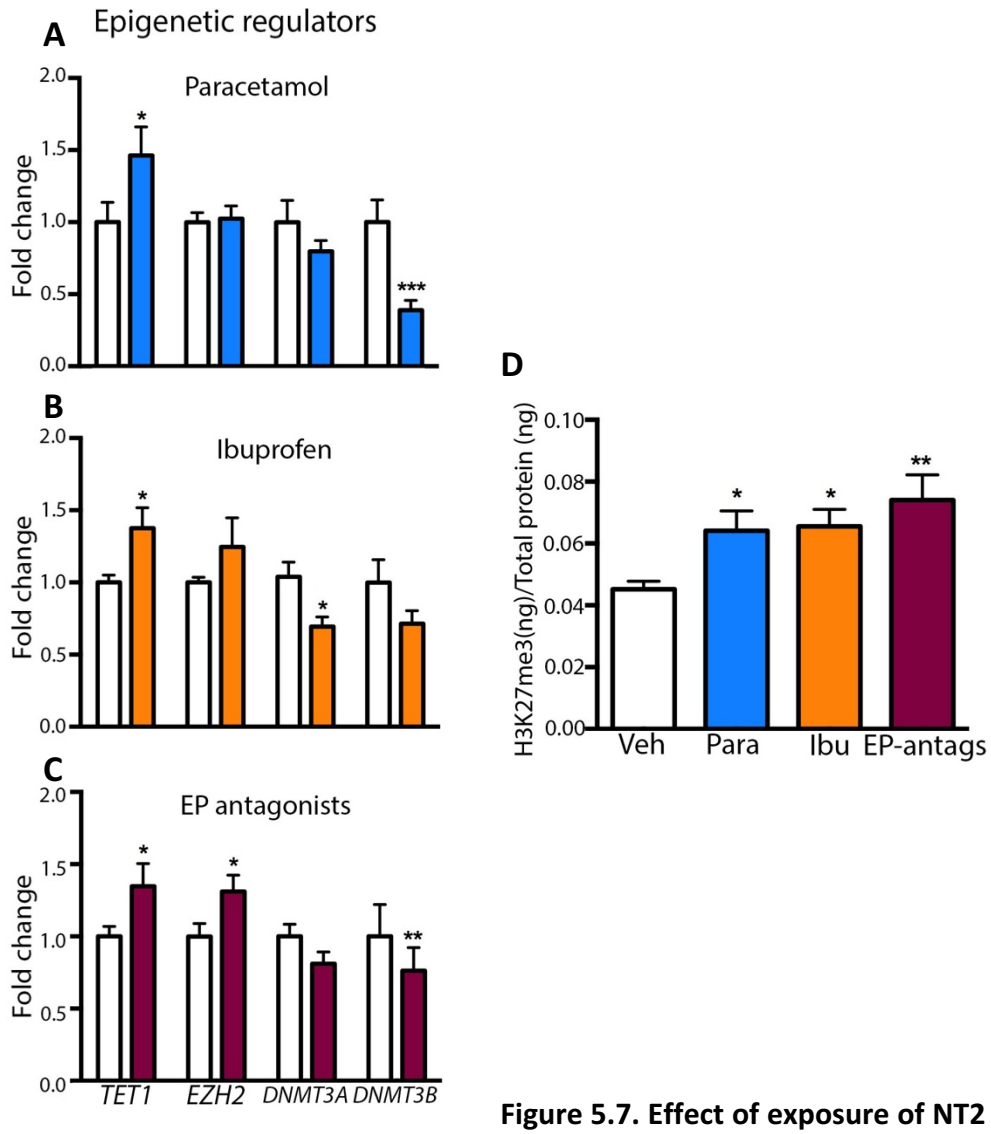


Figure 5.7. Effect of exposure of NT2 cells to analgesics or prostaglandin E_2 receptor antagonists on mRNA expression of epigenetic regulatory genes and overall H3K27me3 levels. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with vehicle (Veh), paracetamol (Para; 10-50µM), ibuprofen (Ibu; 10µM) or EP2+EP4 antagonists [EP-antags (10µM L-161,982; EP2 antagonist + 10µM PF04418948; EP4 antagonist)]. Results show mRNA expression relative to the vehicle complementary DNA (open bars) for the epigenetic regulatory genes *TET1*, *EZH2*, *DNMT3a* and *DNMTb* (**A-C**) after exposure of NT2 cells to paracetamol (blue), ibuprofen (orange) or EP2+EP4 antagonists (maroon) (n=3). Panel **D** shows relative amount of H3K273 present in the NT2 cells after culture with the different treatments. Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, **p<0.01, ***p<0.001, in comparison with corresponding vehicle or vehicle complementary DNA.

5.3.7. Effect of paracetamol, ibuprofen and EP2+EP4 antagonists on the Wnt4/β-catenin pathway in NT2 cells

As explained in chapter 1, the Wnt4/β-catenin pathway is one of the most well-known pathways in gonad development. It is part of the signalling needed for the differentiation of the gonad into an ovary or testis (Clevers & Nusse, 2012). As shown in chapter 3, Wnt4 expression was significantly decreased in fetal rat testes after in vivo exposure to paracetamol or indomethacin (section 3.3.2). Similar analyses were performed on NT2 cells after exposure to paracetamol, ibuprofen or EP2+EP4 antagonists. Results showed that paracetamol and PGE_2 receptor antagonists were able to decrease *WNT4* and β-*CATENIN* expression in NT2 cells after 48h exposure. However, results were not the same for the different doses of paracetamol and the lower dose of paracetamol only reduced β-*CATENIN* expression, while the higher dose of the drug only decreased *WNT4* expression. However, exposure to EP2+EP4 antagonists decreased the expression of both genes (Figure 5.8).

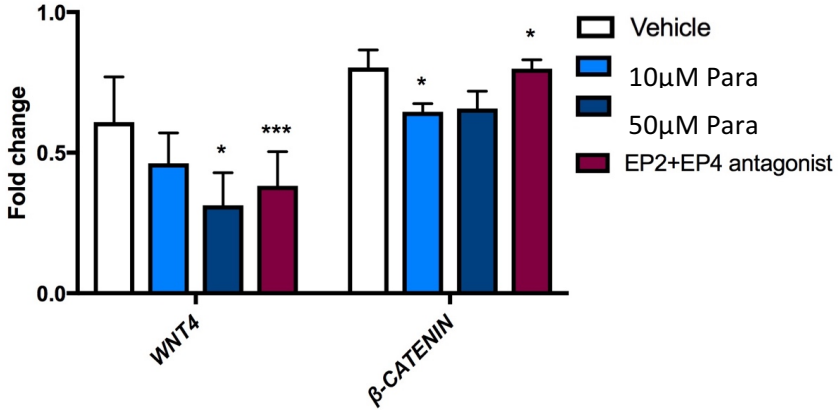


Figure 5.8. Effect of exposure of NT2 cells to analgesics or prostaglandin E_2 receptor antagonists on mRNA expression of *WNT4* and β-*CATENIN*. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with vehicle (Veh), paracetamol (Para; LD=10µM and HD=50µM) or EP2+EP4 antagonists [EP-antags (10µM L-161,982; EP2 antagonist + 10µM PF04418948; EP4 antagonist)]. Results show mRNA expression relative to the vehicle complementary DNA (open bars) for *WNT4* and β-*CATENIN* after exposure of NT2 cells to paracetamol (blue) or EP2+EP4 antagonists (maroon) (n=3). Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, ***p<0.001, in comparison with corresponding vehicle or vehicle complementary DNA.

5.3.8. Effect of EZH2 and JMJD3 inhibitors on NT2 cell number

In this chapter I have shown a wide range of modifications as a consequence of paracetamol, ibuprofen or PGE_2 receptor antagonist exposure of NT2 cells. However, the relationships between these modifications is still unknown. The modifications in the epigenetic machinery (able to modify the expression of certain genes) seen when NT2 cells were exposed to the treatments could be one of the explanations for the other effects seen, such as the decrease in cell number, change in cell cycle or decrease in pluripotency marker expression. Nonetheless, the other possible explanation is that that the other effects seen are the actual cause of the epigenetic modifications. Therefore, the PRC2 complex was altered in NT2 cells in order to compare the effects. To do so, NT2 cells were exposed to either an EZH2 (GSK-126) or a JMJD3 (GSK-J4) inhibitor for 48h before cell number was calculated. Theoretically, if the effects seen in NT2 cells by analgesic exposure are a consequence of alterations in activity of the PRC2 complex, by inhibiting JMJD3, exposed NT2 cells should show similar effects to those seen after analgesic exposure. JMJD3 reduces the levels of H3K27me3 and hence, by inhibiting this enzyme, it should increase the presence of H3K27me3, as the analgesics do (section 5.3.6). On the contrary, by inhibiting EZH2, levels of H3K27me3 should decrease and so, NT2 cells should show the opposite effects to analgesics.

When NT2 cells were exposed to the JMJD3 inhibitor for 48h, cell number was highly reduced (Figure 5.9A), similar to the percentage of dead cells (Figure 5.9B). NT2 cells exposed to EZH2 inhibitor for 48h showed a small decrease in cell number (Figure 5.9A) and no effect on dead cells (Figure 5.9B). The cell cycle after the exposure of NT2 cells to either inhibitor was also studied. No effect could be seen when NT2 cells were exposed to the EZH2 inhibitor. However, exposure to the JMJD3 inhibitor modified the NT2 cell cycle such that there was a significant increase in the proportion of cells in G0/1 phase, an increase in cells in S phase and a significant reduction in the proportion of cells in G2/M (Figure 5.9C).

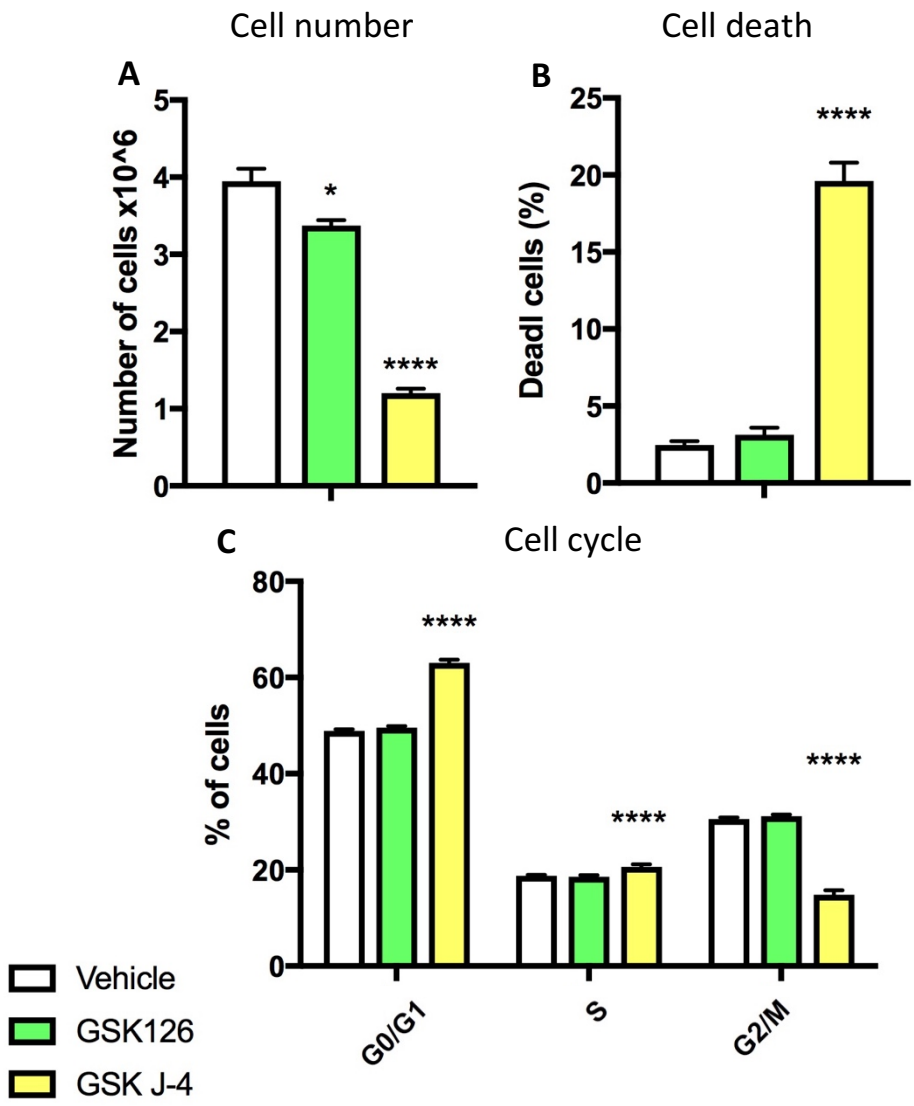


Figure 5.9. Effect of exposure of NT2 cells to EZH2 and JMJD inhibitors on cell number, cell death and the cell cycle. NT2 cells ($2x10^5$; n=3-4) were cultured for 48h in medium supplemented with either vehicle, EZH2 inhibitor (GSK 126; 0.3µM) or JMJD3 inhibitor (GSK J4; 4µM). The NT2 cell number for the EZH2 inhibitor (Green) and JMJD3 inhibitor (Yellow) is shown in panel **A.** Panel **B** shows the % of dead cells in NT2 cells after exposure to the different treatments. Panel **C** represents the % of cells in the different cell cycle stages with the different treatments. Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, ****p<0.0001 versus corresponding vehicle-exposed group.

5.4. Discussion

Having shown consistent effects of analgesics in the different rat and human fetal gonad models, the primary aim of the studies in this chapter was to perform deeper analyses related to the mechanisms involved and whether these were able to explain the effects seen. For that reason, NT2 cells, a GC-like cellular model, were used. This model provides an easier and more tractable way to study the effects of paracetamol and ibuprofen on human fetal GCs. Despite the differences between actual human fetal GCs and NT2 cells, the latter is a cellular model that shows similar characteristics to human fetal GCs (Hoei-Hansen et al., 2004), as explained in section 5.3.1. One advantage of the NT2 cell model is that it allows the study of a relatively uniform population of GC-like cells, unlike whole gonad studies or gonad cultures, where GC are a small proportion of all the cells present in the sample.. The easy access to a large number of NT2 cells makes it easier to expose the cells to a wider variety of treatments, which allowed me to study the effect of PGE_2 receptor inhibitors and different doses of paracetamol as well as perform rescue experiments. Moreover, NT2 cell cultures provided enough material (i.e. RNA, proteins, cell count, etc.) to perform experiments such as flow cytometry and ELISA analysis, that would be otherwise challenging using human fetal gonads, because of the lack of availability of human fetuses.

For all these reasons, NT2 cells provided a suitable human model to study the mechanistic pathways altered by paracetamol, ibuprofen or PGE_2 receptor antagonists, having in mind the different studies performed in rat and human fetal gonads and the results obtained. New approaches were also taken into account, such as a more detailed study of the normal PGE_2 pathways and if paracetamol, ibuprofen or PGE_2 receptor antagonists could alter them.

5.4.1. Decrease in NT2 cell number by Paracetamol, ibuprofen and EP2+EP4 antagonists

In light of the results in chapter 4 showing a reduction in GC number after exposure of human fetal gonads to human relevant doses of paracetamol or ibuprofen, it was studied if similar effects on cell number could be seen in the NT2 cell model. 48h exposure to human relevant doses of paracetamol or ibuprofen reduced the NT2 cell number compared with the vehicle exposed cultures. As expected, reduction in NT2 cell number after exposure to both analgesics followed similar trends to those found after exposure of 1st and 2nd trimester human fetal gonads to analgesics, as shown in chapter 4. Moreover, these reductions in NT2/GCs as a consequence of analgesic exposure are similar to the experiments performed in rodents, including the ones from this lab showing a decrease in fetal GC number after in utero exposure to paracetamol or indomethacin in rats (Dean et al., 2016) and other published data showing a decrease in fetal follicle number after paracetamol exposure of female mice in fetal life (Holm et al., 2016). Furthermore, when NT2 cells were exposed to EP2+EP4 antagonists, similar patterns of change in cell number were found, giving strength to the hypothesis that paracetamol and ibuprofen effects on GC number (and NT2 cells) are a consequence of alterations in the PGE_2 pathway.

5.4.2. Prostaglandin E2 receptor agonists can rescue the decrease in NT2 cell number by paracetamol.

The similar patterns of NT2 cell number reduction after exposure to paracetamol or ibuprofen or to EP2+EP4 antagonists is not enough proof to confirm that both analgesics are affecting cell number by altering the PGE_2 pathway. Even if both analgesics and EP2+EP4 antagonists decreased NT2 cell number, these treatments could be affecting different pathways resulting in similar phenotypes. One of the best ways to study if a treatment is affecting a specific pathway is altering that pathway in the opposite direction to see if the phenotype can be rescued. This principle was used to study the effect of paracetamol on NT2 cell number and its possible relationship to the PGE_2 pathway, by using PGE_2 receptor agonists (EP2+EP4

agonists). The ability of the EP2+EP4 agonists to prevent the paracetamol-induced reduction in NT2 cell number when exposed to paracetamol at the same time, gives more support to this hypothesis that paracetamol is acting via the PGE_2 pathway.

Similar experiments using ibuprofen and EP2+EP4 agonists were not performed. Ibuprofen's effect on the PGE_2 pathway are well known (Rainsford, 2003; Burian & Geisslinger, 2005), but even if it is reasonable to hypothesize that EP2+EP4 agonists would be able to prevent the NT2 cell number reduction as a consequence of ibuprofen exposure, both analgesics are not acting exactly on the same pathways. As shown with the study of the NT2 cells after paracetamol/ibuprofen exposure, both analgesics affected differently the cell cycle of the cells (section 5.3.4.1), revealing an example of mechanistic differences between both analgesics. Hence, whether EP2+EP4 agonists can rescue the NT2 cell reduction by ibuprofen should be addressed in future experiments.

5.4.3. Paracetamol, ibuprofen and EP2+EP4 antagonists affect NT2 cell death

The reduction in NT2 cell number as a consequence of paracetamol, ibuprofen or EP2+EP4 antagonist exposure can derive mainly from two different reasons: an increase in cell death or a decrease in cell proliferation. In my experiments, among the human relevant doses of paracetamol and ibuprofen used, only paracetamol (lower dose) exposure significantly increased NT2 cell death. However, this increase after paracetamol exposure was not big enough to argue that it could be the cause of the reduced NT2 cell number, although it is difficult to extrapolate from findings on cell death at one moment in time (the end of culture). The high increase in NT2 cell death after exposure to the higher dose of paracetamol was significantly more pronounced, in keeping with the higher reduction in NT2 cell number found after this treatment. The increase in cell death after the higher dose of paracetamol indicates that paracetamol at this concentration is probably detrimental to NT2 cells. This response to different doses of paracetamol has also been reported in in vivo

experiments, where high doses of paracetamol are also detrimental, especially for liver cells, while lower doses are considered to be safe (McGill et al., 2012). Similar to the lower dose of paracetamol, exposure to EP2+EP4 antagonists caused a significant increase in NT2 cell death, but again, such a small change that it seems unlikely to explain the reduction in NT2 cells seen after exposure to EP2+EP4 antagonists.

5.4.4. NT2 cell cycle is affected by paracetamol, ibuprofen and EP2+EP4 antagonists

Following the logic from the previous section in explaining the decrease in NT2 cell number after exposure to paracetamol, ibuprofen or EP2+EP4 antagonists, if it cannot be explained by an increase in cell death, the reduction in NT2 cell number might be explained by a reduction in cell proliferation. In this case, NT2 cell proliferation was studied indirectly by studying their cell cycle after exposure to the different treatments.

Interestingly, the effects found on the cell cycle by the three different treatments were different. The analysis by flow cytometry showed that paracetamol exposure shifted the NT2 cells towards the G2/M stage, reducing the proportion of cells in G0/G1 compared with the cells treated with vehicle. An increased number of cells in G2/M is normally associated with an increased number of cells, which was not the case with NT2 cells cultured with paracetamol. These results suggest that NT2 cells are arrested in G2/M as a consequence of paracetamol exposure, which results in a decreased number of cells dividing and hence, a decrease in final cell number at the end of the culture. The increase in the proportion of cells in the synthesis phase is significant, but not really pronounced, making it difficult to know the possible consequences and reasons of this increase.

The effects of ibuprofen and EP2+EP4 antagonists on the NT2 cell cycle did not follow the same pattern as paracetamol. While Ibuprofen did not show any significant

modification in the proportion of cells in the different cell cycle stages compared with vehicle, EP2+EP4 antagonist exposure, contrary to paracetamol, reduced the proportion of cells in G2/M. This result suggests a lower percentage of cells dividing, which could explain the reduction in NT2 cell number seen after EP2+EP4 antagonist exposure. However, this reduction in NT2 cells in G2/M was not compensated by an increase in the proportion of cells in other cell cycle stages. This could be because the increase in the number of cells in other stages is divided within those stages and significance would be probably found with an increase in the number of experiments performed.

These results on cell cycle stage in NT2 cells are the first difference in effects that were found between paracetamol/ibuprofen and EP2+EP4 antagonists, but also the first difference between paracetamol and ibuprofen. The reasons for these differences can be many, starting with the different pathways that these analgesics are altering. This is especially relevant in the case of paracetamol, where regardless of the multiple researches performed, its exact effect on cells is still unclear (Anderson, 2008).

Paracetamol studies on cell cycle by qPCR revealed interesting modifications in markers of the different cell cycle stages. However, there was not a dose response comparing the higher with the lower dose of paracetamol with any of the genes. The down regulation of one of the markers of G0/G1 by paracetamol exposure of NT2 cells correlates with the decrease in the proportion of cells in that cell cycle stage. The Up-regulation of the studied markers for G2/M after paracetamol exposure also correlates with the increased proportion of NT2 cells in G2/M when cultured with the same analgesic. The studied markers for the transition between mitosis and G0 were down-regulated after paracetamol exposure, which fits with the hypothesis of fewer cells actually finishing the mitosis stage and starting the cycle again. However, not all of the cell cycle markers studied followed the expected patterns. For example, CCNE1, one of the markers of G0/G1 was not modified after paracetamol exposure.

Similar negative results were found for the studied markers for the synthesis and G2 phases, when NT2 cells were exposed to paracetamol. The analysis of the cell cycle is a complex type of study. With the information obtained, it is difficult to discuss why not all the studied genes followed the expected patterns. Nonetheless, all together, the study of the cell cycle by specific markers reinforces the hypothesis of arrested NT2 cells in G2/M phases as a consequence of culturing them in the presence of paracetamol.

The comparison of studies on cell cycle by qPCR as a consequence of EP2+EP4 antagonists also revealed differences when compared with paracetamol. When NT2 cells were exposed to EP2+EP4 antagonists for 48h, only *HIST1H4B* was significantly modified amongst the genes studied. These results, along with the different proportion of cells found in the cell cycle phases, when comparing paracetamol and EP2+EP4 antagonists, further suggests that paracetamol affects the cell cycle via a different route than EP2+EP4 antagonists. Further experiments should be addressed to understand these differences and what other pathways paracetamol might be affecting.

5.4.5. NT2 cell pluripotency gene expression is affected by paracetamol, ibuprofen and EP2+EP4 antagonists

As would be predicted, the present studies in NT2 cells showed a reduced expression of pluripotency markers when these cells were exposed to paracetamol or ibuprofen for 48h. Both analgesics reduced the expression of *OCT4*, showing similar results to the previous experiments by this lab that had shown reduced *Oct4* expression in rat fetal testis when they were in utero exposed to paracetamol or indomethacin (Dean et al., 2016). A recent publication has also shown that ibuprofen reduced the expression of germ cell pluripotency markers such as *OCT4* and *LIN28* in fetal human testis explants (Ben Maamar et al., 2017). Moreover, the studies of this thesis also showed that NT2 cells exposed to paracetamol, but not ibuprofen, reduced the

expression of *NANOG* and *AP2γ,* two other important GC pluripotency markers (Hoei-Hansen et al., 2005; Jørgensen et al., 1995).

EP2+EP4 antagonist exposure of NT2 cells had a similar effect to paracetamol, reducing the expression of *OCT4*, *AP2γ* and *NANOG.* These results are consistent with previous studies showing that exposure of mouse embryonic stem cells to an EP2 antagonist, a cell relatively similar to gonocytes and NT2 cells, led to down-regulation of pluripotency genes, such as *Oct4* and *Nanog* (Yun et al., 2012). Additionally, there is abundant other evidence of a fundamental role of PGE_2 in altering the differentiation status of a diverse variety of stem cells, as explained in section 1.5.3 (Wong et al., 2016; Zhang et al., 2014; Caron et al., 2016). However, this is the first time that the role of PGE_2 on the pluripotency state was studied in fetal human GC-like cells, such as the NT2 cells.

The consequences of a possible alteration in the pluripotency state of GC is unclear. However, some of the possibilities involve a reduction in GC number later on in development or an increased risk of testicular GC cancer (TGCC). A reduction in GC pluripotency markers could be a consequence of premature differentiation of these GCs, which could prematurely switch off proliferation. As mentioned in section 4.4.2, fetal GC are more proliferative at early stages of development and their proliferation rate is decreased around week 10 of gestation in humans (Mamsen et al., 2011). If analgesics are accelerating normal fetal GC development, as has been suggested before (Dean et al., 2016), this could decrease the final pool of GC present in the gonads. Because of the lack of proliferation in female GCs after the maximum peak reached during fetal development (Morita & Tilly, 1999), the consequences of analgesics on GC pluripotency stage could be far more detrimental for females than males (Dean et al., 2016).

Another possible consequence of alteration of the pluripotency state of the NT2 cells or of fetal GC is the possible relationship with TGCC. Arrested differentiation of fetal GC is now accepted as the most likely pathway for the development of TGCC in

humans, the commonest malignancy amongst young men (Mitchell et al., 2014). However, the reduction in expression of pluripotency genes in NT2 cells seen in this chapter and in vivo in GC in the rat fetal testis (Dean et al., 2016), as a consequence of analgesic exposure, appears to be in the opposite direction to what is thought to underlie the origins of TGCC. Hence, having in mind that the results of the effects of analgesics on pluripotency markers was studied in NT2 cells, a GC cell line, these results could show an alternative link between analgesics and TGCC and it is that these drugs might have some efficacy in the treatment of TGCC. However, the present results should be complemented with more specific studies to evaluate this other option, such as culture of TGCC samples with paracetamol or ibuprofen, or studying the mechanisms via which these drugs alter the pluripotency markers of GCs.

5.4.6. NT2 cell epigenetic machinery is affected by paracetamol, ibuprofen and EP2+EP4 antagonists

Study of the effects of analgesic exposure of NT2 cells on epigenetic regulators gave the expected results. Both paracetamol and ibuprofen significantly increased the expression of *TET1* in NT2 cells, although the expression of *EZH2* was not modified. These results revealed similar patterns to those found in the different rat models shown in chapter 3, where analgesics were able to increase the expression of *Tet1*. Results in rats also showed an increase in *Ezh2* expression, but this pattern was not seen in NT2 cells exposed to paracetamol or ibuprofen. The increased expression of *TET1* and *EZH2* by EP2+EP4 antagonists in NT2 cells reinforces the hypothesis that analgesics affect the epigenetic machinery of GCs (and NT2 cells) via the PGE_2 pathway.

The fact that different models (in vivo rat exposure, in vitro rat culture and NT2 cells) show similar effects of analgesic exposure on epigenetic regulatory gene expression is suggestive of a conserved mechanism. However, it does not give information about the importance of this effect on the actual physiology of the GC. This is the reason

why the results showing an increase in the total H3K27me3 present in NT2 cells after exposure to the different treatments is key to start understanding the implications of exposing GC to analgesics. This is the first experiment showing an actual interaction between analgesics and the epigenetic machinery in GCs. The consequences of this increase in H3K27me3 on the GCs still need to be properly addressed. However, an increase in H3K27me3 could result in decreased expression of important genes that are key during fetal gonad development (Di Croce & Helin, 2013), especially as numerous 'poised' genes, expressing high H3K27me3, have been identified in fetal GC during their pluripotent phase (Lesch & Page, 2014). It is established that the process of permanent switching specific genes either on or off via H3K27me3 is a part of normal fetal GC development (Sachs et al., 2013; Lesch et al., 2013). Therefore, anything that perturbs these mechanisms of normal GC development is likely to have fetal GC consequences. Furthermore, the regulation of fetal GC development by PGE_2 seen in the different models implies a key conserved role for PGE_2 in the physiological regulation of these events. This is further supported by the published literature showing PGE_2 regulation of similar processes in other cells/tissues/tumours (Yun et al., 2012; Wong et al., 2016; Nakanishi & Rosenberg, 2013).

5.4.7. WNT4/β-CATENIN pathway is affected by paracetamol and EP2+EP4 antagonists in NT2 cells

The decrease in WNT4 and β-CATENIN expression seen after 48h exposure to the different doses of paracetamol on NT2 cells followed a similar pattern to that seen after in utero analgesic exposure in the rat fetal model (Section 3.3.2). These results using NT2 cells reinforce the hypothesis that analgesics, and PGE_2, affect the WNT4/β-catenin pathway. As explained in section 1.3.2.1, this pathway is fundamental for normal fetal gonad development, especially for sex differentiation (Clevers & Nusse, 2012).

Furthermore, the similar results on wnt4/β-catenin expression seen after EP2+EP4 antagonist in NT2 cells are the first ones showing that $PGE_2$and the Wnt4/β-catenin pathway are related. Further analysis should be done in the future to address the exact relationship between these two pathways and to investigate if the Wnt4/β-catenin pathway is directly or indirectly regulated by PGE_2, as well as its importance for fetal gonad development.

The consequences of alterations in the Wnt4/β-catenin pathway are difficult to address, particularly with the present results showing just a small reduction in gene expression and no demonstration that these effects altered protein expression level. Complete deletion of *Wnt4* has been related to trans-differentiation of granulosa cells into Sertoli cells (Lavery et al., 2012; Nicol & Yao, 2015). In a human context, mutations in *WNT4* are usually associated with a spectrum of disorders affecting the genitalia, such as such severe hypospadias, or abnormal development of gonadal morphology (Jordan et al., 2001; Domenice et al., 2004; Parma et al., 2006).

5.4.8. Effect of EZH2 and JMJD3 inhibitors on NT2 cell number

The decrease in NT2 cell number after 48h exposure to an EZH2 inhibitor was unexpected because it was a change in the opposite direction to what had been hypothesised. Because of the lack of knowledge of the actual consequences of an increase of H3K27me3 on NT2 cells, it is perhaps difficult to predict the cell behaviour after their exposure to an EZH2 inhibitor. When NT2 cells were exposed to analgesics, they showed an increase in H3K27me3 and a decrease in proliferation. Following these results, cell proliferation (i.e. cell number after culture) might be regulated by H3K27me3 (and hence, analgesic treatments), although these results seen in NT2 cells exposed to analgesics could be completely independent. However, based on these studies, if cell proliferation is negatively controlled by H3K27me3, then inhibition of EZH2 should decrease H3K27me3 and increase proliferation, producing an effect contrary to that of paracetamol, ibuprofen or EP2+EP4 antagonists. This hypothesis has been proved in previous studies using mammary stem cell lines

exposed to an EZH2 inhibitor, which showed that some specific mammary stem cell types increased proliferation and cell number, although the contrary was seen with other cell types (Wu & Crowe, 2015). Another study exposing mouse fetal gonads to a much higher concentration of GSK126, the same EZH2 as the studies of this thesis, showed no detrimental effect of the drug on the GCs, although they did not study the effect on GC proliferation (Prokopuk et al., 2017). It is important to recall that when NT2 cells were exposed to PGE_2 agonists, cell number did not increase as has been shown in other cell types (Yun et al., 2009). This lack of increase in cell number after EP2+EP4 agonists could mean that NT2 cells, and potentially fetal GCs, might already be at a peak of proliferation which cannot be increased further by PGE_2. Furthermore, if GC/NT2 cell proliferation is being driven by PGE_2, it implies that this drive is already maximal. Nonetheless, NT2 cells showed a small decrease in cell number when exposed to an EZH2 inhibitor, but this decrease was not supported by an increase in percentage of dead cells or any change in the NT2 cell cycle. The decrease in cell number could result from other secondary effects of the inhibition of EZH2 that cannot be addressed with the current information. Such effects may be entirely independent of PGE_2 also.

The inhibition of JMJD3 should stop the demethylation of H3K27me3 and hence, maintain or increase the levels of this histone marker. If proliferation is negatively controlled by PRC2/ H3K27me3, the JMJD3 inhibitor should mimic the effects of analgesics and the EP2+EP4 antagonists; the finding that the JMJD3 inhibitor did decrease NT2 cell number is thus consistent with this prediction. However, the decrease in cell number when NT2 cells were exposed to JMJD3 inhibitor was much greater than with the analgesics or the PGE_2 receptor antagonists, and its effect may have been as a result of inducing NT2 cell death. In this respect, its effect could perhaps be equated with that of high dose paracetamol which also caused a substantial increase in NT2 cell death, similar to that seen after exposure to a JMJD3 inhibitor, although the possibility of non-specific toxicity by such exposure cannot be ignored. Nonetheless, the changes in NT2 cell cycle were different between the

exposure to high dose paracetamol (cells shifted to the G2/M phase) and the exposure to the JMJD3 inhibitor (cells shifted to the G0/G1 phase). It is difficult to compare the concentration of JMJD3 inhibitor with the action that paracetamol, ibuprofen and EP2+EP4 antagonists might have on PRC2 activity. However, previous experiments with the same dose of JMJD3 inhibitor using other cell types did not report this level of cell death (Mathur et al., 2017). A few publications have used JMJD3 inhibitors as a treatment for different cancers, such as glioma or lymphomas or leukaemia (Mathur et al., 2017; Hashizume et al., 2014; Ntziachristos et al., 2014). Judging these publications, there is the reasonable possibility that, as NT2 cells are tumour derived cells, the JMJD3 inhibitor could have a similar effect to that shown in these publications. Whether normal fetal GCs would be affected in a similar way by JMJD3 inhibitors should be addressed in future experiments. These effects of JMJD3 suppression on NT2 cell number reveal a potential use of JMJD3 inhibitors as a possible therapeutic treatment for TGCC, which would need further research.

5.5. Conclusion

Chapters 3 and 4 provided some background about analgesic effects on rat and human fetal gonads, which were consistent and showed how these consequences can be translated within different models. The next aim was to identify an intrinsic mechanism that was able to explain the consequences of analgesic exposure, such as the effects on the epigenetic machinery or on genes important for fetal gonad development, as explained in chapter 3, or the reduction in GC number, as shown in chapter 4.

In this chapter I focused on the NT2 cell culture model to study in detail the possible consequences of analgesic exposure on a human fetal GC-like cell in culture. Despite the differences between NT2 cells and normal fetal GCs, NT2 cells showed similar patterns of response to analgesic exposure as did the other rat and human fetal gonad models used. The decrease in NT2 cell number in response to paracetamol and ibuprofen exposure is similar to that seen for fetal GC number in 1st and 2nd

trimester human fetal gonads (chapter 4). Again, NT2 cells showed alteration in their pluripotency gene expression after analgesic exposure, similar to that shown in previous studies in rats (Dean et al., 2016) and in human (Ben Maamar et al., 2017). The increased expression of the epigenetic regulators *TET1* and *EZH2* seen in NT2 cells exposed to paracetamol or ibuprofen also mimics the results seen in fetal rat gonad cultures (Chapter 3).

Moreover, all of the analgesic-related modifications seen in NT2 cells could be reproduced by inhibiting the PGE_2 pathway by EP2+EP4 antagonists. Prevention of the paracetamol-induced decrease in NT2 cell number by EP2+EP4 agonists is the final evidence to highlight the hypothesis that the majority of the effects induced by paracetamol and ibuprofen on fetal GCs are a consequence of their effect on the PGE_2 pathway. However, the differences in the cell cycle studies after exposure to paracetamol, ibuprofen and EP2+EP4 antagonists emphasize that we still do not understand the actual mechanisms of effect of paracetamol. Even if paracetamol is altering the PGE_2 pathway, there are other routes that can be affected in GCs whose consequences are still completely unknown.

The present findings in the NT2 cells reinforce the results from the rat and human models discussed in chapters 3 and 4, and suggest that PGE_2 may play a fundamental across-species role in the development and/or differentiation of fetal GC, including their proliferation, their pluripotency state and their epigenetic regulatory machinery.

Chapter 6: Final discussion

6. Final discussion

The aims of this thesis were to establish the consequences of exposure to paracetamol, ibuprofen and indomethacin, often taken by pregnant women (Jensen et al., 2010; Philippat et al., 2011; Werler et al., 2005), in fetal gonads. Within fetal gonads, the main focus of the research was the study of the effects of these analgesics on GCs. One of the priorities was to identify the mechanism of action of paracetamol, ibuprofen and indomethacin that is able to explain the various effects seen in previous studies, as well as those found during this project. The main changes seen as a consequence of analgesic exposure, were a reduction in GC number (probably via reduced proliferation), and reduction in gene expression of GC pluripotency markers and of factors involved in regulation of the epigenetic machinery.

Different models were used in this thesis, including human and rat systems. Rat models entailed in utero exposure to analgesics (paracetamol or indomethacin), or hanging drop fetal gonad cultures, which were optimized as part of this thesis. Human models involved 1st trimester human gonad culture (testis and ovary), 2nd trimester fetal testis xenografts and a more tractable system, a fetal GC-like cellular model known as NT2 cells.

6.1. Paracetamol and indomethacin affect the expression of important genes related in fetal gonad and germ cell development

Chapter 3 was focused on the rat models and continued one area of the research that the lab had previously been studying, involving the in vivo exposure to analgesics of pregnant rats. These experiments had shown that paracetamol and indomethacin reduced fetal GC number and altered their normal development (Dean et al., 2016). The experiments were performed on gonads exposed in utero to paracetamol or indomethacin with a developmental age between e15.5-e17.5. These studies revealed that both analgesics can alter the expression of genes involved in RA

signalling, such as an increase in mRNA expression of the RA synthesiser *Aldh1a1, in* e17.5 testes. However, deeper studies revealed that other genes normally modified by RA (RA receptors and metabolisers) were not altered, leaving some doubt as to whether analgesics really do target the RA pathway effectively. An increase in RA by analgesics would be consistent with previous analyses in the lab, which showed an increase in *Stra8,* which is regulated by RA*,* in e17.5 and e18.5 fetal ovaries (Dean et al., 2016). Increased levels of RA could potentially alter the normal onset of meiosis, which has been shown to increase apoptosis in GCs (MacLean et al., 2007).

The expression of other genes affected by in vivo exposure to analgesics in e17.5 testes was studied, including *Foxo3, Notch2, Epiregulin, EGFR* and *Inha,* which are involved in normal GC development (Feng et al., 2014a; John et al., 2008; Xu & Gridley, 2013). Moreover, changes in *Wnt4* expression were also found, a key factor in gonad development (Elzaiat et al., 2016). It is important to point out that the majority of these genes, which are physiologically more important in the ovary, were studied in e17.5 testes because of the lack of samples from females, which had been allocated to other experiments within the lab. However, the mechanisms altered by the analgesics could potentially be affected in the ovary as well, leading to similar results. Further analysis using ovarian samples should be done to address this possibility.

As explained in detail in section 3.4.3, alterations in expression of *Foxo3, Notch2, Epiregulin, EGFR* and *Inha,* could have detrimental effects on normal GC development or produce GC loss (Castrillon et al., 2003; Hsieh et al., 2007; Romero & Smitz, 2010). Wnt4, whose expression was also reduced by paracetamol or EP2+EP4 antagonists in the NT2 model, works together with Rspo1 and β-catenin to actively inhibit *Sox9* expression during ovary fetal development (Elzaiat et al., 2016). However, the mRNA expression of *Rspo1* and *β-catenin* were not reduced in rat fetal testes after in utero exposure to analgesics. A decrease in *Wnt4* expression in the ovary might interfere with normal gonad development by increasing *Sox9* expression, which can induce trans-differentiation of granulosa cells into Sertoli cells

(Lavery et al., 2012). In the fetal testis, *Wnt4* is expressed at low levels but its complete inhibition/ablation affects testis formation, decreased proliferation and increased apoptosis of male GC (Boyer et al., 2012; Das et al., 2013). In this context, one of the factors for sex determination during fetal development is another PG, PGD_2. Translocation of SOX9 to the nucleus in NT2 cells is dependent on cAMP and PKA, and is mediated by PGD_2 (Malki et al., 2005). Studies in mice lacking PGD_2 synthetase (Pgds), showed decreased levels of *Sox9* transcript in Sertoli cells (Moniot et al., 2009). Within the mice context, increased levels of PGD_2 resulted in partial masculinization of female embryonic gonads in culture (Adams & McLaren, 2002). PGD_2 and PGE_2 have differentiated roles in cell function. However, may of the pathways they affect are similar, such as the modification in cAMP and PKA (Ricciotti & FitzGerald, 2011), raising the possibility of a certain degree of similarity on the effects of PGE_2 and PGD_2 on fetal gonad development.

Together, these alterations in expression of genes that play important roles in gonad or GC development after in utero exposure to analgesics, confirm that analgesics are affecting the developing fetal gonad, although it is difficult to discuss the final consequences of these changes. One of the limitations of these studies in this in vivo rat model is that all of the modifications found were at the gene expression level, which may not be reflected in similar protein expression changes. What is clear from these results is that the analgesic-induced modifications in specific pathways of gonad/GC development are not dramatic, and hence it is difficult to find definitive consequences or detrimental effects resulting from the altered expression of these genes. Moreover, the changes found could be incidental, even though they were consistent.

6.2. Paracetamol and ibuprofen exposure reduce GC/NT2 cells number

One of the most consistent results reported in this thesis is the reduction in GC number after analgesic exposure, as this change was seen in all different human fetal gonad models studied in this thesis. My lab had already shown in previous studies

that in utero exposure to paracetamol or indomethacin reduced GC number in fetal rat ovaries and testes (Dean et al., 2016). Another publication reported similar reduction in ovarian GC number after mice were exposed to different doses of paracetamol in utero (Holm et al., 2016). However, the results of this thesis are the first to reveal a similar effect of analgesics on GC number in human fetal gonads. The analgesic-induced reduction in GC number was seen across the gestational range examined in human fetal gonads (Chapter 4) and in the NT2 cell model (Chapter 5), all of which involved exposure to human-relevant therapeutic doses of paracetamol or ibuprofen (Albert et al., 2013; Janssen & Venema, 1985; Mazaud-Guittot et al., 2013). The analgesic-induced reduction in GC number was shown in 1st trimester fetal ovaries and testes cultured in hanging drops and exposed for 7 days to paracetamol or ibuprofen; these studies showed a more pronounced loss of GC in the ovary than in the testis. A similar GC reduction was also found in 2nd trimester human fetal testis xenografts exposed for 1 or 7 days to paracetamol or to ibuprofen (7 day exposure only), a model system which has been shown to recapitulate normal development of human fetal testis GC (Mitchell et al., 2010). GC number reduction seen in 2nd trimester human fetal xenografts as a consequence of paracetamol/ibuprofen exposure was most pronounced for the AP2γ+ gonocytes compared to the more differentiated pre-spermatogonia (MAGEA4+), suggesting a possible effect of analgesics targeted to a specific GC subpopulation. Furthermore, NT2 cells, which are more akin to gonocytes than to pre-spermatogonia, showed a similar reduction in cell number after exposure for 48h to human-relevant concentrations of paracetamol or ibuprofen.

One of the possible explanations for the reduction in fetal GC/NT2 cells seen in the different systems used in this thesis and previous publications as a consequence of analgesic exposure is a decrease in cell proliferation. This was further studied showing a decrease in the percentage of proliferative gonocytes in 1st trimester human fetal testes and ovaries after 7 days exposure to paracetamol or ibuprofen. 2nd trimester samples did not show a significant reduction in proliferative GCs after

exposure to either analgesic, although it did show a pattern of reduction with the different treatments. The lack of a statistically significant reduction in GC proliferation after analgesic exposure of 2nd trimester samples may be due to the low number of experimental replicates in these experiments. Another possible explanation for the differences in the reduction of proliferation found between 1st and 2nd trimester human fetal samples exposed to analgesics is the lower proliferation of pre-spermatogonial GCs, which are more common in 2nd trimester human fetal testes (Mamsen et al., 2011), as a lower basal proliferation would make it more challenging to find small changes in proliferation caused by the analgesics. The study of changes in cell proliferation in the NT2 model as a consequence of analgesic exposure was performed by studying their cell cycle. These results showed important differences in cell cycle behaviour between NT2 cells exposed to paracetamol, ibuprofen or EP2+EP4 antagonists, revealing that paracetamol seemed to arrest NT2 cells in the G2/M phase, which could explain the reduction in cell number after paracetamol exposure, while ibuprofen did not show any difference and EP2+EP4 antagonists reduced the number of cells in G2/M. Further studies on the expression of markers for the cell cycle stages confirmed that paracetamol exposure of NT2 cells increased the expression of markers of G2/M and decreased the expression of G1/M markers. The reason for the differences found between paracetamol, ibuprofen or EP2+EP4 antagonist exposure was not addressed in this thesis. However, the main possible explanation for these results is that paracetamol is affecting the cell cycle via a route not related to PGE_2 action.

The findings on analgesic-induced reduction in GC number in the human fetal testis and ovary in each model system used, together with the reduction in GC number in rat fetal gonads after analgesic exposure in vivo (Dean et al., 2016) and the reduction in NT2 cell number, demonstrate the robust nature of the effect, raising the possibility of similar effects of analgesics on fetal GC in human pregnancy. A reduction in GC number in fetal gonads is relevant because the majority of pregnant women are exposed to at least one analgesic during pregnancy. However, the

consequences of this reduction in GC number in humans is difficult to address. The studies by this lab showed how a reduction in GC number in rats during fetal development as a consequence of analgesic exposure, reduced adult ovary weight and the fertility of females, measured by pups per litter, yet no effect was seen in males (Dean et al., 2016). This is probably because female GC can no longer proliferate after their entry into meiosis, which happens from week 9 of gestation in humans (Bendsen et al., 2006; Gondos et al., 1986), meaning that GC reduction during or after this gestational age might have long-term consequences for the oocyte reserves and thus for the total reproductive lifespan (Nelson et al., 2013). The lack of phenotype seen in adult male rats exposed in utero to analgesics is probably because of postnatal compensation of GC number, as their GC continue to proliferate after birth as well as from puberty through to adulthood, while female GC proliferation is stopped during fetal development (Kocer et al., 2009; Tingen et al., 2009); it seems likely that a similar male-female difference would apply in humans. Moreover, other research not related to analgesics, but which studied changes in fetal GC number, have shown that a decrease in fetal GCs might have a detrimental effect on female ovarian lifespan, leading to premature ovarian failure (Hirshfield, 1994; Song et al., 2015). A reduction in GC number in human female fetuses could thus result in fertility consequences in adulthood. This possibility could be addressed in humans by studying the relationship between paracetamol or ibuprofen use in pregnancy and the levels of AMH in the blood of resulting female offspring, as AMH levels have been correlated with oocyte reserve under certain conditions (Dewailly et al., 2014), or ultimately in age at menopause.

6.3. Paracetamol and ibuprofen exposure affect the prostaglandin pathway and germ cell pluripotency in fetal gonads

An important aim of the research of this thesis was to study the mechanisms via which analgesics affected GCs. The mechanism studied in this thesis was the prostaglandin E_2 pathway, which is known to be altered by paracetamol and ibuprofen (Aminoshariae & Khan, 2015; Anderson, 2008; Hecken et al., 2000). This

has also been proved by this lab, by showing that analgesic exposure in utero of fetal rats reduced PGE_2 levels in the fetal gonads (Dean et al., 2016). Moreover, fetal GC in both rat and human express PGE_2 receptors (Bayne et al., 2009; Dean et al., 2016). Regarding the capacity of analgesics to reduce GC/NT2 number discussed above, one explanation for this effect is the possible alteration of the PGE_2 pathway. PGE_2 has been shown to be related to mechanisms of stem cell proliferation in a variety of different cell types (Hoggatt et al., 2009; Wang et al., 2013; Xia et al., 2012; Yun et al., 2009). NT2 cells, which share a number of features with stem cells, were exposed to PGE_2 receptor antagonists for 48h, which resulted in a similar reduction in cell number to that induced by paracetamol or ibuprofen exposure. Moreover, the paracetamol-induced decrease in NT2 cell number was prevented by exposing these cells to EP2+EP4 agonists at the same time as paracetamol. These results using NT2 cells cultured with EP2+EP4 antagonists or agonists demonstrate that alteration of the PGE_2 pathway by analgesic exposure, is probably responsible for the changes in GC/NT2 numbers and gene expression changes. Regarding the latter, the present results have shown that PGE_2 antagonism induced similar gene expression modifications in NT2 cells as analgesics induced in both rat (fetal gonad cultures and in vivo) and human (NT2 cells). The effect of PGE_2 in regulating GC/NT2 proliferation thus seems to be conserved across species. This effect has not only been seen in rodents as in this study and other publications (Dean et al., 2016; Holm et al., 2016), but has also been shown in less advanced organisms, such as the giant fresh water prawn, where variation in PGE_2 affects ovarian maturation by shortening the length of the ovarian cycle, and also affects oocyte proliferation (Sumpownon et al., 2015).

Focusing on other well-known effects of PGE_2 on different cell types, GC/NT2 pluripotency gene expression was studied. The analysis of the expression of GC pluripotency markers in NT2 cells exposed to analgesic or PGE_2 antagonists showed a reduction in *OCT4*, *AP2*γ and *NANOG* expression. These results are consistent with our previous analysis showing a reduction in Oct4 expression in fetal GC of male rat fetuses exposed to analgesics in vivo (Dean et al., 2016). Moreover, previous studies

showed that *Oct4* and *Nanog* expression were reduced in mouse embryonic stem cells exposed to an EP2 antagonist (Yun et al., 2012), and there is plenty of other evidence demonstrating a fundamental role of PGE_2 in the regulation of cancer cell and stem cell differentiation status and proliferation in different cell types (Jones et al., 2010; Nandi et al., 2017; Wang et al., 2013; Xia et al., 2012). In a more relevant context, human fetal testis explants exposed to ibuprofen showed a reduction in GC pluripotency marker expression including *OCT4* and *LIN28A* (Ben Maamar et al., 2017).

The consequences of the analgesic-induced alteration in GC differentiation in a human context might be related to TGCC, the commonest malignancy amongst young men, as arrested fetal GC differentiation is now accepted to be the primary cause (Mitchell et al., 2014). However, the reduction in GC pluripotency gene expression as a consequence of analgesic exposure seen in NT2 cells and in the other models in published research, appears to be in the opposite direction to what is accepted to occur with the origin of TGCC. Other possible consequences of the alteration in GC pluripotency markers can be earlier differentiation of GCs. This idea was already proposed in previous studies in the lab on fetal rats exposed in vivo to analgesics (Dean et al., 2016). A possible consequence of earlier differentiation of fetal GCs is that more advanced GCs (pre-spermatogonia) are less proliferative than earlier GCs (gonocytes) (Mamsen et al., 2011). If analgesics caused earlier differentiation of GCs, it could be a possible explanation for the reduction in proliferation seen in human fetal GCs as a consequence of paracetamol or ibuprofen exposure and the consequential reduction in GC/NT2 cells. Moreover, this idea of advanced GC development as a consequence of analgesic exposure would square well with the results suggesting that analgesics affect gonocytes more than pre-spermatogonia. This is because a reduction in proliferation would probably affect more the gonocytes subpopulation, as it is a more proliferative cell type. However, this should also be addressed in future experiments by studying in more detail the proliferation status of the different GC subpopulations following analgesics

exposure. Moreover, a higher sample number would help to clarify differences between the reductions in proliferation comparing gonocytes and pre-spermatogonial GCs.

My studies also focused on specific pathways of action potentially affected by analgesics (and potentially by PGE_2). These showed that mRNA expression of the specificity proteins *Sp1* and *Sp2* were modified in fetal gonads by paracetamol or indomethacin exposure in vivo in the rat as also were different epigenetic regulators, such as *TET1*, *EZH2*, *DNMT3A* and *DNMT3B,* expression of which was modified by paracetamol and ibuprofen or indomethacin in both the rat and NT2 models. SP proteins are involved in the regulation of a wide variety of genes related to fundamental cellular processes, including cell growth, apoptosis or differentiation. Moreover, SP proteins are gene specific, cell specific and are known to regulate some gonad genes (Huang et al., 2012; Tan & Khachigian, 2009). Modification of the expression of *Sp1* and *Sp2* could potentially alter the expression of genes important in fetal gonad development and explain some of the phenotypes seen in the present studies. However, further study of the relationship between SPs and their effect on the developing gonad should be performed. This could be done by, for example, using SP inhibitors, which would allow the study of the consequences of alteration of SP proteins independently from the rest of the effects of analgesics.

6.4. Paracetamol and ibuprofen exposure affect the epigenetic machinery in fetal gonads

The effects of analgesic exposure on mRNA expression of epigenetic regulatory genes was studied in both rat fetal gonad models (in vivo and in vitro) and in NT2 cells, and showed some general patterns replicated in all models. The expression of *TET1* was increased in all the models (in vivo and in vitro fetal rat and NT2 cells) after exposure to the different analgesics and this was also seen in many of the cases for *EZH2* expression, although the treatment-induced increase in expression of *EZH2* was not consistent between models. Further experiments showed similar patterns in *TET1*

and *EZH2* expression when rat fetal gonad cultures or NT2 cells were exposed to EP2+EP4 antagonists. TET1 and EZH2 are both epigenetic regulators related to the PRC2 complex, and hence increased expression of these genes should in theory result in an increased presence of the histone methylation marker H3K27me3; this hypothesis was confirmed in further experiments in NT2 cells exposed to paracetamol, ibuprofen or to EP2+EP4 antagonists. At least one earlier publication had already related PGE_2 action to H3K27me3 (Arosh et al., 2015). However, the present studies are the first to show that analgesics/PGE_2 receptor inhibitors can modify H3K27me3 in a fetal GC-like cell type. It is now fully accepted that H3K27me3 is highly expressed and dynamically regulated in fetal GC and is important in the regulation of important factors in fetal GC differentiation and development (Sasaki & Matsui, 2008; Seki et al., 2007). However, the studies in this thesis do not delineate the genes affected by global changes in H3K27me3 in GC/NT2 cells. This could be addressed in the future by doing ChIP-seq to find distinct profiles of gene expression as a consequence of analgesic exposure.

The expression of other epigenetic regulatory genes potentially modified by analgesics or PGE_2 antagonists, such as the methyltransferases enzymes DNMT3A and DNMT3B, was also analysed. The expression of both those genes was decreased by analgesics or EP2+EP4 antagonists in the different models of rat and human, although the changes were not reproduced in all cases. Previous studies have already shown alteration of DNMTs expression and/or DNA methylation by PGE_2 in different cell types, including tumour cell lines (Arosh et al., 2015; Huang et al., 2012; Venza et al., 2012; Xia et al., 2012). DNMTs expression is correlated with DNA methylation levels and, as mentioned in the literature review (Section 1.4.2), fetal GC undergo extensive de- and re-methylation during fetal and early postnatal development (Guo et al., 2014; Sasaki & Matsui, 2008). Hence, analgesic exposure might have an impact on this process by altering DNMTs expression.

Furthermore, as explained in chapter 1 (Section 1.4.2.5), there is cross-talk between DNA methylation and H3K27me3. Hence, finding modifications of both markers after analgesic or EP2+EP4 antagonist exposure is in keeping with this. Different studies have shown that when the level of, for example DNA methylation, is decreased, the other marker (H3K27me3) is increased, probably because both epigenetic modifications are markers of gene repression (Hagarman et al., 2013). Alterations in both epigenetic markers (DNA methylation and H3K27me) as a consequence of modifications in the PGE_2 pathway has been reported (Arosh et al., 2015). However, that publication showed an alteration of H3K27me3 in the opposite direction to that shown in these studies of NT2 cells exposed to paracetamol, ibuprofen or EP2+EP4 antagonists. There are various possibilities to explain this difference between these earlier results in endometriotic cells and the results in this thesis. First, the studies were carried out in different models, as Arosh et al used an endometriotic cell line whereas that used in this thesis is a fetal GC-like cell line. Therefore the different results could be a reflection of the different cell types behaving differently to alterations in the PGE_2 pathway, although, so far, the modifications shown in different publications using different cell types after altering the PGE_2 pathway have shown a high degree of agreement (Jones et al., 2010; Nandi et al., 2017; Sumpownon et al., 2015; Wang et al., 2013; Yun et al., 2009; 2012). The findings of different studies revealing an opposite cross-talk between DNA methylation and H3K27me, showing that an increase in the levels of one factor decreases the levels of the other (Hagarman et al., 2013; Wu et al., 2010), fits with the results found in this thesis, but not with those seen in Arosh et al (Arosh et al., 2015). Moreover, some recent studies focused on the PRC2 complex on mouse fetal gonads showed how by inhibiting Ezh2, H3K27me3 levels were reduced. However, when important genes for gonad development usually regulated by H3K27me, such as some genes from the Hoxb locus, were studied after exposure to the Ezh2 inhibitor, these genes did not show an altered expression (Prokopuk et al., 2017). Hence, it is important to consider that the results in this thesis showing an increase in *TET1/EZH2* expression after analgesic exposure do not provide all the facts to allow us to sensibly interpret the

results. All the roles of the PRC2 complex in any cell, but specially in GCs is still far to be fully understood and there can be interactions within other cellular mechanisms that are difficult to explain.

One important topic to discuss about the epigenetic modifications derived from analgesic exposure is the possibility of these modifications being transmitted to the next and subsequent generations. Previous publications have already shown how environmental modifications can alter some epigenetic mechanisms, which might have transgenerational consequences (Carone et al., 2010; Radford et al., 2014). The possible transgenerational consequences of analgesic exposure was already raised in the previous studies by my lab showing that the F2 females derived from either females or males exposed to paracetamol while fetuses (F1), exhibited smaller ovaries and reduced follicle numbers during puberty and adulthood (Dean et al., 2016). The results of that publication were not truly inter-generational, as only the F2 generation was studied. However, the detrimental effects of F1 analgesic exposure on the F2 are likely to be caused by epigenetic modifications and raises the possibility of trans-generational effects that should be addressed in the future. This should be done by planning experiments involving more generations, or using other models easier for transgenerational studies, such as *C. Elegans* or *Drosophila Melanogaster.* Regardless of the experiments used, the modifications of the epigenetic machinery by analgesics demonstrated in the rat fetal gonad culture and the NT2 cell cultures are the first indication of what possible epigenetic mechanisms are able to explain the transmission of the detrimental effects of analgesics to later generations.

The findings shown in this thesis can only be viewed as providing circumstantial support for the possibility that analgesics might induce epigenetic modifications in fetal GC in human pregnancy. However, the similar results found in the different model systems provide methods that can be used in the future to investigate this possibility further. Moreover, the robust results in this thesis indicate that PGE_2

probably plays a fundamental and conserved role in the modification of important elements of the epigenetic machinery, similar to that shown already for other tissues (Arosh et al., 2015; Venza et al., 2012). This also raises the possibility that analgesic use/exposure could have other additional effects (beneficial or adverse) to the ones shown here, which in the future could be used for therapeutic purposes.

The studies presented in this thesis have many different limitations. The first and probably most important one is that none of the models accurately recreates in-vivo human conditions. The human xenograft and culture models recreate important aspects of normal human fetal development, such as GC proliferation and differentiation (Jorgensen et al., 2015; Mitchell et al., 2010; Spade et al., 2014). The xenograft model is probably the most physiological system we can use for the study of human fetal gonads as it is the most similar to in utero exposure. Regarding the NT2 cells, these are not a true fetal GC line: indeed none exists. However, NT2 cells are fetal GC-like cells and express similar markers to fetal GCs, as they derive from TGCC precursor cells, which are believed to originate from fetal GCs (Hoei-Hansen et al., 2004). Despite all the limitations found in the different models, it is important to consider the consistency of the effects found in GC/NT2 cells after analgesic exposure. The similar modifications in gene expression in human and rats (Including in vivo and in vitro), suggest that the mechanisms modified by analgesics/PGE_2 are probably highly conserved and therefore, they are likely to follow similar patterns in vivo in humans.

During this thesis, I have focused on the effects of analgesics on fetal GC. However, as explained in section 1.2.3, previous research has shown effects of analgesics on Leydig cell function and subsequently on testosterone production. GC and Leydig cells can communicate with each other (Spradling, Fuller, Braun, & Yoshida, 2011), so it is likely that there is a degree of impact in GC affecting other cell types and perhaps vice versa. For this reason, the effect of analgesics in fetal GCs should not be studied in isolation, because anything that disturbs the fetal gonad environment has

potential to have indirect effects on other gonadal cell types. In that sense, other possible studies that could be done using the human fetal gonad samples is the analyses of hormone production after analgesic exposure. This has been studied already in previous analyses on human samples (Mazaud-Guittot et al., 2013; van den Driesche et al., 2015), which have shown a decrease in testosterone production as a result of analgesic exposure, but further studies using our models could be addressed to confirm these results and to find possible correlations with the other phenotypes seen involving GC consequences.

The results presented in the thesis have important implications for the common use of analgesics by pregnant women (Philippat et al., 2011; Werler et al., 2005). One of the most concerning direct effects is the reduction in GC number shown in the human fetal gonad cultures and xenografts. As discussed above, the decrease in GC number in testes is potentially compensated for postnatally, as shown in the studies using rat models (Dean et al., 2016). However, the same studies showed how the reduction of GC in fetal ovary can result in health/reproductive implications. Yet, the experiments performed in this thesis cannot evaluate completely the consequences of analgesics exposure in pregnant woman. After taking account of the results of this thesis and previous experiments (Dean et al., 2016; Holm et al., 2016), it would be appropriate to discuss the need for studies of girls born to mothers in whom analgesic use in pregnancy has been carefully (prospectively) recorded. AMH levels could be measured as an indicator of ovarian reserve in the female offspring. However, it is important to consider that AMH levels are quite variable (Dewailly et al., 2014) and therefore, large numbers would be needed. Considering the number of analgesic-induced adverse effects that are now being proposed, not only from the reproductive point of view, but from a wide variety of other consequences, a study that aimed to explore multiple endpoints in resulting offspring (male and female), from reproductive to behavioral, etc., would be justified and, indeed, looks to be essential if the 'real risks' of using analgesics in pregnancy are to be established rather than the theoretical risks that are being discussed now. A study of this proportions

would need the coordination of different teams of specialists and probably from different hospitals.

6.5. Suggestions for future studies

The studies shown in this thesis cover a broad spectrum of the study of the consequences of fetal exposure to analgesics, especially focused on GCs. This thesis showed important results confirming that at least some of the phenotypic changes described as a consequence of analgesic exposure of fetal gonads result from alterations in the PGE_2 pathway. Furthermore, the results presented here showed that analgesics/PGE_2 can modify the GC pluripotency state and the epigenetic machinery in GC/NT2 cells. However, despite the variety of models used and the robust similar results within these models, the analyses shown lack a deeper research regarding the actual consequences of the modifications seen.

One of the most important results seen is probably the alteration in global H3K27me3 in NT2 cells after exposure to analgesics or EP2+EP4 receptor antagonists, because of the importance of this histone repressive marker in regulating the expression of different key genes in GC/gonad development. However, whether the alteration in H3K27me3 is a cause or a consequence of the rest of the phenotypes seen has still to be addressed. The use of JMJD3 and EZH2 inhibitors to modify the PRC2 complex and hence H3K27me3, seemed to be an interesting approach in theory, although it did not induce the expected effects. However, deeper analysis could be done by using other regulators of the PRC2 complex or either using the same inhibitors, but at different concentrations. Moreover, a deeper analysis should be done to see what are the effects of altering H3K27me3 by studying genes regulated by this histone repressive marker and investigating whether any alterations are produced by analgesic exposure. The study of the modifications of different genes/pathways as a consequence of the alteration of H3K27me3 could be investigated in different models. However, it should probably be started in NT2 cells, as they are a more tractable system. Moreover, it produces more material for doing more advanced

experiments, such as RNA sequencing. Once the experiments have revealed some consequences of alteration of H3K27me in NT2 cells, more physiologically relevant models could be used, such as the human fetal gonad culture/xenograft models and /or in vivo in rats to see whether or not they are consistently modified by alteration of H3K27me3.

In addition to the epigenetic regulators, other mechanisms could be altering the expression of different genes after analgesics exposure, such as SP1 or SP2. Hence, it would be interesting to do a global study of gene expression as a consequence of analgesic or EP2+EP4 antagonist exposure. This could be studied in the NT2 cells by doing RNA-sequencing after the exposure. These experiments would provide a great source of information and would help to find, not only modified genes, but potentially to find the mechanisms by which they were altered as well. Any identified genes/pathways could then be investigated in the human fetal gonad culture/xenograft models and /or in vivo in rats to see whether or not they are consistently modified by analgesic and/or EP2/EP4 antagonist exposure. Moreover, if compounds are available that can experimentally manipulate these pathways independent of analgesic exposure, then new routes of investigation will be opened.

Other experiments could be performed in order to complete the information found so far. It would be important to do some studies to investigate whether analgesic exposure induces modifications at the protein level that would correlate with the gene expression changes shown using qPCR. Another valuable set of experiments would be to rescue analgesic effects. The use of EP2+EP4 agonists to rescue the decrease in NT2 cell number was helpful to prove the role of the PGE_2 pathway in the effects seen after paracetamol exposure. However, it should also be investigated if EP2+EP4 agonists can rescue ibuprofen effects. Furthermore, studies on gene expression should be done to see if EP2+EP4 agonists could rescue the modifications seen in gene expression, such as the GC pluripotency markers. Moreover, this

experiment should be repeated in more physiological models, such as the human fetal gonad cultures or the xenografts model.

In the same line of inquiry, another side of the research that would be worth exploring would be a deeper research on the human fetal testis xenograft to delineate the long-term consequences of analgesics. The xenograft model offers the possibility of exposing the gonads for 1 week to an analgesic but keeping the samples inside the host mice for a longer period to study if the consequences (or which consequences) of the analgesic exposure are compensated or not over time. This would be interesting because, as shown in previous experiments by my lab in rat models, the reduction in GCs in male fetuses produced by analgesics can be compensated for postnatally (Dean et al., 2016).

The main focus of this thesis was the study of the consequences of analgesic exposure on fetal gonad development. However, some of the results revealed possible therapeutic use of the analgesics and the JMJD3 inhibitor. As shown in this thesis, the exposure of NT2 cells to paracetamol, ibuprofen or EP2+EP4 antagonists resulted in a decrease in cell number and a decrease in the expression of GC pluripotency markers. Moreover, exposure to JMJD3 inhibitor produced a high detrimental effect on theNT2 cells. These experiments in NT2 cells, which derived from TGCC, showed a possible use of these different exposures as a potential treatment for TGCC. Using the hanging drop or the xenograft systems, TGCC tissue could be exposed to analgesics, PGE_2 receptor antagonists or JMJD3 inhibitors to explore the potential use of these exposures for the treatment of TGCC. This experiment would be feasible, because of the relatively easy access to TGCC from patients, and the similarities between the experiments already performed on human fetal gonads and these new set of experiments would make them easier to perform.

6.6. Summary

The data presented in this thesis has shown how exposure of human fetal testes or ovaries to human therapeutically relevant doses of paracetamol or ibuprofen causes a consistent decrease in fetal GC number. Analgesics produce other modifications as well, such as changes in gene expression and modifications of the epigenetic regulatory machinery. These effects of analgesic exposure were robust, were conserved among the different rat and human model systems, and are probably a consequence of alterations of the PGE_2 pathway. Even if these results can probably be translated to human pregnancy, this has to be considered with caution. Nonetheless, the robust nature of the results of this thesis and the possible involvement of a conserved mechanism, support the recommendation of using analgesics during human pregnancy with caution and consideration of risks and benefits. Furthermore, following the effects of short-term exposure of paracetamol, analgesic use should be recommended for the shortest period necessary to manage symptoms. The findings reported in this thesis are part of a growing body of evidence for a range of effects of analgesics during pregnancy that have been reported in different publications during the last decade (Hurtado-Gonzalez & Mitchell, 2017; Kristensen et al., 2011; Lind et al., 2017; Mazaud-Guittot et al., 2013; van den Driesche et al., 2015). These findings, together with those presented in this thesis, and coupled with the high prevalence of analgesic use by pregnant women, create concern about the use of over the counter analgesics by pregnant women. However, there is a need for better and more physiological human models, and also the need for properly designed prospective studies in order to find more definitive associations between analgesic use during pregnancy and abnormalities in the offspring that might be predicted from the various model systems.

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Analgesic use in pregnancy and male reproductive development

Pablo Hurtado-Gonzalez and Rod T. Mitchell

Purpose of review
Male reproductive disorders are common and increasing in incidence in many countries. Environmental factors (including pharmaceuticals) have been implicated in the development of these disorders. This review aims to summarize the emerging epidemiological and experimental evidence for a potential role of in-utero exposure to analgesics in the development of male reproductive disorders.

Recent findings
A number of epidemiological studies have demonstrated an association between in utero exposure to analgesics and the development of cryptorchidism, although these findings are not consistent across all studies. Where present, these associations primarily relate to exposure during the second trimester of pregnancy. In vivo and in vitro experimental studies have demonstrated variable effects of exposure to analgesics on Leydig cell function in the fetal testis of rodents, particularly in terms of testosterone production. These effects frequently involve exposures that are in excess of those to which humans are exposed. Investigation of the effects of analgesics on human fetal testis have also demonstrated effects on Leydig cell function. Variation in species, model system, dosage and timing of exposure is likely to contribute to differences in the findings between studies.

Summary
There is increasing evidence for analgesic effects on the developing testis that have the potential to impair male reproductive function. However, the importance of these findings in relation to human-relevant exposures and the risk of male reproductive disorders remain unclear.

Keywords
analgesics, male reproduction, NSAIDS, paracetamol, testis

INTRODUCTION

Development of the male reproductive system is dependent on normal formation and function of the testis during fetal life. Failure of normal development may result in disorders that manifest in the neonatal period (cryptorchidism and hypospadias), or in adulthood (testicular cancer and poor semen quality) [1,2▪].

In humans, cryptorchidism occurs in 1–4.6% of newborns, although it will often resolve naturally leading to a prevalence of ~1% at 1 year [3,4]. Cryptorchidism is associated with an increased risk of testicular cancer, the commonest malignancy amongst young men, which is believed to arise from aberrant development of a population germ cells, known as gonocytes, during fetal life [5]. Cryptorchidism is also associated with impaired spermatogenesis resulting in a 30–60% risk of infertility in adulthood [6]. Hypospadias is also a relatively common disorder occurring in approximately 0.2–0.6% of male newborns [7]. The term Testicular Dysgenesis Syndrome is frequently used to describe the association of these disorders as a result of events that occur during fetal life and their relationship with deficient androgen production or action [8]. Indeed, a critical period from embryonic day (e)15.5–e18.5, known as the ''masculinization programming window'' (MPW), has been described in fetal rats during which reduction in androgen production or action leads to the subsequent development of cryptorchidism and hypospadias [9▪▪]. A similar period of sensitivity has been postulated to

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KEY POINTS

- Analgesics are used by the majority of women during pregnancy.
- Use of mild-analgesics during pregnancy has been associated with an increased risk of cryptorchidism in some studies.
- Exposure to analgesics results in Leydig cell effects in a number of experimental animal models of fetal testis development.
- The importance of human-relevant in-utero exposures to analgesic in relation to male reproductive health remains to be determined.

occur in the first trimester in humans based in part on the timing of divergence in anogenital distance (AGD; an indicator of fetal androgen exposure) between males and females [10].

These common male reproductive disorders have increased in incidence over recent decades indicating that in addition to genetic abnormalities, environmental factors such as life style, diet, and chemical (including pharmaceutical) exposures are likely to play a role in their development [1,11▪]. Recently, there has been an increasing literature on the potential role of in utero exposure to analgesics, including paracetamol and nonsteroidal anti-inflammatory drugs (NSAIDS; e.g., ibuprofen or aspirin), on male reproductive development. This review will describe the emerging epidemiological and experimental evidence in relation to analgesic exposure and its potential effects on male reproductive development.

ANALGESIC USE DURING PREGNANCY

Women are generally advised to avoid taking medications during pregnancy where possible. However, despite this, the majority of women take one or more analgesics such as paracetamol or NSAIDS, at some point during pregnancy [12]. A large Danish study ($n = 46{,}500$) reported analgesic use in 55% of pregnant women [13], whereas a US study ($n = 10{,}533$) reported 65% of pregnant women used paracetamol (15% in combination with ibuprofen) [12]. A smaller ($n = 895$) French study reported an even higher frequency of analgesic use (81%) in pregnant women [14]. Furthermore, the overall consumption of analgesics has increased significantly in the majority of European countries during the past 20 years [15▪]. These analgesics are able to cross the placenta and hence have the potential to cause direct effects on the fetus [16–18]. It is not ethical to test the effects of analgesics on pregnant women directly and hence the evidence for associations between analgesic use during pregnancy and the development of male reproductive disorders derives from a combination of epidemiological and experimental studies conducted largely over the past two decades.

EPIDEMIOLOGICAL STUDIES RELATING TO ANALGESIC EXPOSURE AND DEVELOPMENT OF MALE REPRODUCTIVE DISORDERS

Association between analgesic use and cryptorchidism

A significant association between the overall use of mild analgesics during pregnancy and cryptorchidism in the offspring has been demonstrated in three studies with adjusted odds ratio (OR) of 1.93 (confidence interval [CI]: 1.03–3.62) [19▪], 2.12 (CI: 1.17–3.83) [20], and 2.30 (CI: 1.12–4.73) [21▪▪]. Another study did not demonstrate a significant association (OR: 1.1; CI: 0.31–3.6) [14] (Table 1). In two of the studies that demonstrated an association this only reached statistical significance for analgesic use during the second trimester with no significant association during the first trimester [20,21▪▪]. Interestingly, Kristensen *et al.* describe data on two separate Scandinavian populations. The significant associations were restricted to the Danish cohort, whereas in the Finnish cohort, there were no statistically significant associations [13]. Differences between cohorts may relate to variations in methodology, prevalence of cryptorchidism, or study power. Duration of exposure may also be important with a significant association between prolonged (>2 weeks) use of mild analgesics during pregnancy (includes first and second trimester) and cryptorchidism (OR: 2.47; CI: 1.02–5.96) [21▪▪]. Several of these studies have also investigated the effects of specific agents on the development of cryptorchidism.

Paracetamol exposure and risk of cryptorchidism

Three studies investigating associations between paracetamol use during pregnancy and cryptorchidism have described adjusted OR > 1.0; however, these do not reach statistical significance (Table 1) [13,19▪,20]. In one of these studies, exposure during the second trimester was significantly associated with cryptorchidism (OR: 1.89; CI: 1.01–3.51), similar to their results for mild analgesics overall [20], whilst this was not the case in the other studies

Table 1. Association between analgesic exposure during pregnancy and male reproductive disorders

	Study period	Cohort	Publication	Gestational period	Analgesics adjusted OR (95% CI)	Paracetamol adjusted OR (95% CI)	Ibuprofen adjusted OR (95% CI)	Aspirin adjusted OR (95% CI)
Cryptorchidism	1996–2002	47,400	Jensen *et al.*, 2010 [13]	Pregnancy[b]	n/a	1.33 (1.00–1.77)	0.88 (0.64–1.19)	1.18 (0.93–1.49)
				First trimester	n/a	0.94 (0.75–1.17)	n/a	n/a
				Second trimester	n/a	1.17 (0.89 –1.54)	n/a	n/a
	2002–2006	3,184	Snijder *et al.*, 2012 [20]	Pregnancy	n/a	n/a	n/a	n/a
				First trimester	0.94 (0.36–2.46)	1.38 (0.52–3.64)	n/a	n/a
				Second trimester	2.12 (1.17–3.83)	1.89 (1.01–3.51)	8.93 (1.84–43.24)[a]	
	1997–2001	491	Kristensen *et al.*, 2011 [21] (Danish cohort)	Pregnancy[b]	1.43 (0.73–2.79)	1.337 (0.70–2.55)	1.82 (0.50–6.61)	2.22 (0.86–5.76)
				First trimester	1.48 (0.66–3.34)	1.61 (0.66–3.90)	n/a	5.60 (1.83–17.1)
				Second trimester	2.30 (1.12–4.73)	1.97 (0.94–4.12)	4.59 (1.10–19.0)	3.76 (1.15–12.3)
	1997–1999	1463	Kristensen *et al.*, 2011 [21] (Finnish cohort)	Pregnancy[b]	0.74 (0.35–1.57)	n/a	n/a	n/a
				First trimester	0.77 (0.26–2.27)	n/a	n/a	n/a
				Second trimester	1.21 (0.53–2.76)	n/a	n/a	n/a
	2003–2006	903	Philippat *et al.*, 2011 [14]	Pregnancy[b]	1.10 (0.31–3.6)	n/a	n/a	n/a
	1987–1990	6,699	Berkowitz & Lapinski. 1996 [19]	Pregnancy	1.93 (1.03–3.62)	n/a	n/a	n/a
Hypospadias	2002–2006	3,184	Snijder *et al.*, 2012 [20]	First trimester	2.05 (0.64–6.58)	2.24 (0.60–8.32)	1.65 (0.21–13.08)[a]	
				Second trimester	0.53 (0.12–2.34)	0.54 (0.12–2.41)	n/a	n/a
	1997–2007	5851	Lind *et al.*, 2013 [24]	First trimester	n/a	1.00 (0.80–1.10)	1.20 (1.00–1.30)	1.30 (0.90–1.70)
	1982–1989	56,037	Correy *et al.*, 1991 [25]	First trimester	n/a	n/a	n/a	3.50 (1.40–8.80)
	1959–1965	50,282	Slone *et al.*, 1976 [23]	First trimester	n/a	n/a	n/a	No association
	1997–2004	14,915	Hernandez *et al.*, 2012 [22]	First trimester	n/a	n/a	No association	

[a]Applies to ''other'' painkillers including NSAIDS.
[b]Refers to exposure during first and/or second trimester of pregnancy.

[13,21▪▪]. Timing of exposure is also likely to be important based on the evidence for an MPW in rodents, postulated to occur between 8 and 14 weeks in humans [9▪▪]. Analysis of data from 8 to 14 weeks demonstrated a hazard ratio (HR) of 1.14 (0.97–1.34), which was significant for exposure > 4 weeks duration; OR: 1.38 (CI: 1.05–1.83) [13]. A second study also demonstrated a significant association OR: 2.78 (1.13–6.84) following prolonged (>2 weeks) exposure to paracetamol, similar to that described for mild analgesics in general [21▪▪]. Again, this association was restricted to the Danish and not the Finnish cohort.

NSAID exposure and risk of cryptorchidism

The association between cryptorchidism and exposure to NSAIDS, for example, ibuprofen and aspirin, has also been investigated (Table 1) [13,20,21▪▪]. No significant associations were demonstrated for overall use of ibuprofen or aspirin during pregnancy [13,21▪▪]. However, Kristensen *et al.* [21▪▪] demonstrated a significant increase in cryptorchidism following exposure to ibuprofen (OR: 4.59; CI: 1.10–19.0) and aspirin (OR: 3.76; CI: 1.15–12.3) during the second trimester. A similar significant association between exposure specifically during the second trimester has also been demonstrated in relation to ''other'' analgesics (i.e., analgesics excluding paracetamol) [20].

These results may indicate the importance of simultaneous use of more than one analgesic during pregnancy. The use of ≥ 2 agents was associated with a significant increase in the risk of cryptorchidism (OR: 7.72; CI: 2.09–28.6) in one study [21▪▪], whilst a second study did not demonstrate a significant association (OR: 1.07; CI: 0.82–1.40) [13].

Association between analgesic exposure and risk of hypospadias

The majority of studies investigating analgesic use during pregnancy and the incidence of hypospadias have not shown significant associations (Table 1) [20,22,23▪]. A study assessing analgesic exposure from one month prior until 4 months after conception showed a significantly increased risk of hypospadias for ibuprofen (OR: 1.20; CI: 1.00–1.30), whereas no association was reported for paracetamol or aspirin [24]. Another study reported a significant association between the use of aspirin and hypospadias (OR: 3.5; CI: 1.4–8.8) [25].

Analgesic exposure and anogenital distance

Cryptorchidism and hypospadias are associated with a reduction in androgen production or action during fetal life [9▪▪]. AGD has been shown to be a reliable and robust measure of fetal androgen exposure in rodents [13,20,21▪▪] and reduced AGD has been associated with cryptorchidism and hypospadias in humans [26,27]. It should be emphasized that measurement of AGD in humans can be technically challenging and it is important that those performing the measurements are sufficiently skilled to avoid inter- and intra-observer bias. A recent study investigated 1027 mother-child pairs, recruited from 10 to 27 weeks of gestation, to determine the association between analgesic exposure in mothers and AGD in the offspring at 3 months of age [28▪▪]. No association was found between exposure to paracetamol or NSAID and AGD; however, exposure to a combination of paracetamol and ''other'' analgesics (including NSAIDS) was associated with reduced AGD_{AS} (ano-scrotal AGD; 32.3 vs. 36.2 mm; $P = 0.03$) but not for AGD_{AP} (ano-penile AGD) which may relate to technical issues in conducting these measurements. In addition, this group included relatively small numbers ($n = 20$). Further investigation of the association between AGD and in utero exposure to analgesics are warranted.

Taken together, the epidemiological evidence indicates that there may be an association between in utero exposure to analgesics, particularly during the second trimester, and cryptorchidism. The evidence for such an association with hypospadias is less convincing. There are a number of limitations to these studies relating to obtaining accurate information regarding the dosage, timing, and duration of analgesics exposure. This is particularly important for retrospective studies in which recall bias is a potential limitation [19▪,20].

EXPERIMENTAL STUDIES RELATING TO ANALGESIC EXPOSURE AND DEVELOPMENT OF MALE REPRODUCTIVE DISORDERS

Effect of analgesics on Leydig cell function in the fetal testis

Testicular descent requires the action of two hormones produced by Leydig cells, namely testosterone and Insulin-like growth factor 3 (Insl3) [29▪]. Several studies have investigated the effect of analgesic exposure on Leydig cell function in the fetal testis. This includes in vivo, ex-vivo and in vitro model systems of paracetamol (Table 2) and NSAID (Table 3) exposure using rodent and human tissues.

Analgesics and testosterone production

Testosterone production can be measured directly (e.g., serum or intratesticular) or indirectly (e.g.,

Table 2. Leydig cell function following exposure to paracetamol

	Species	Model	Duration (days)	Dose[a]	Age (start of treatment)	Result	Publication
Testosterone	Rat	In vivo	7	350	GD13	↔	Kristensen *et al.*, 2011 [21]
		In vivo	3	350	e13.5	↓ (~40%)	van den Driesche *et al.*, 2015 [30▪▪]
		In vitro	2	1 μmol/L	e14.5	↓ (~50%)	Kristensen *et al.*, 2011 [21]
		In vitro	2	1 μmol/L	e14.5	↓ (~25%)	Kristensen *et al.*, 2012 [31]
	Human	In vitro	1	10 μmol/L	8–12 GW	↔	Mazaud-Guittot *et al.*, 2013 [34]
		Xenograft	7	60	14–20 GW	↓ (~50%)	van den Driesche *et al.*, 2015 [30▪▪]
AGD	Rat	In vivo	7	150	GD13	↓ (~10%)	Kristensen *et al.*, 2011 [21]
		In vivo	6	350	GD13	↔	Axelstad *et al.*, 2015 [33]
		In vivo	3	350	e13.5	↓ (~10%)	van den Driesche *et al.*, 2015 [30▪▪]
	Mouse	In vivo	13	150	GD7	↓ (~15%)	Holm *et al.*, 2015 [32▪]
INSL3	Rat	In vivo	3	350	e13.5	↔	van den Driesche *et al.*, 2015 [30▪▪]
		In vitro	3	100 μmol/L	e14.5	↔	Kristensen *et al.*, 2012 [31]
	Human	In vitro	3	10 μmol/L	10–12 GW	↓ (~40%)	Mazaud-Guittot *et al.*, 2013 [34]

For significant effects (↑/↓), the result shown represents the minimum dose and shortest duration showing significance. For nonsignificant effects (↔), the highest dose and longest duration is shown. e, embryonic day; GD, gestational day; GW, gestational weeks.
[a]Doses are given by mg/kg/day unless otherwise stated.

AGD). Paracetamol exposure has been linked to a reduction of fetal testicular testosterone production in several studies [21▪▪,30▪▪,31] (Table 2). In-vivo exposure of fetal rats to paracetamol (350 mg/kg/d) during the MPW significantly decreased AGD by up to 10% in late fetal life [21▪▪,30▪▪], whereas another study only demonstrated a significant effect on AGD (15% reduction) at 10 weeks postnatally with no effect at 4, 6, or 8 weeks [32▪]. One of these studies also described a significant reduction (40%) in intratesticular testosterone and in mRNA expression of two key steroidogenic enzymes (CYP17a1 and CYP11a1) indicating a potential mechanism for the effect on steroidogenesis [30▪▪]. Another study in rats did not demonstrate an effect of exposure during this time-window on AGD at birth, although there was an effect of exposure to a chemical ''mixture'' that

Table 3. Leydig cell function following exposure to NSAIDS

	Drug	Species	Model	Duration (days)	Dose[a]	Age (start of treatment)	Result	Publication
Tesosterone	Aspirin	Rat	In vivo	7	200	GD13	↓ (~60%)[b]	Kristensen *et al.* 2011 [21]
			In vitro	1	10 μmol/L	e14.5	↓ (~70%)	Kristensen *et al.* 2011,2012 [21,31]
		Human	In vitro	3	100 μmol/L	8–10 GW	←	Mazaud-Guittot, 2013 [34]
			In vitro	3	1 μmol/L	10–12 GW	↔	Mazaud-Guittot *et al.* 2013 [34]
	Indomethacin	Rat	In vivo	3	0.8	e15.5	↔	Dean *et al.* 2013 [36]
			In vitro	1	10 μmol/L	e14.5	↓ (~30%)	Kristensen *et al.* 2012 [31]
		Human	In vitro	2	10 μmol/L	8–12 GW	↑ (~20%)	Mazaud-Guittot *et al.* 2013 [34]
AGD	Aspirin	Rat	In vivo	7	250	GD13	↔	Kristensen *et al.* 2011 [21]
			In vivo	3	150	GD11	↓ (~20%)	Gupta and Goldman, 1986 [35]
	Indomethacin		In vivo	3	1	GD11	↓ (~20%)	Gupta and Goldman, 1986 [35]
			In vivo	3	0.8	e15.5	↔	Dean *et al.* 2013 [36]
Insl3	Aspirin	Human	In vitro	3	10 μmol/L	8–12 GW	↔	Mazaud-Guittot *et al.* 2013 [34]
	Indomethacin		In vitro	3	10 μmol/L	8–12 GW	↔	Mazaud-Guittot *et al.* 2013 [34]

For significant effects (↑/↓), the result shown represents the minimum dose and shortest duration showing significance. For nonsignificant effects (↔), the highest dose and longest duration is shown. e, embryonic day; GD, gestational day; GW, gestational weeks.
[a]Doses are given by mg/kg/day unless otherwise stated.
[b]Only significant after 3 h incubation (150 mg/kg/d—no significant effect).

included paracetamol [33]. In vitro studies have also demonstrated analgesic effects during the MPW. Exposure of e14.5 rat fetal testis to 1 μmol/L paracetamol for 48 h resulted in a 15–50% reduction in testosterone with significant reductions described across a range of doses (0.5–10 μmol/L) at 72 h, whereas a similar approach using culture of human fetal testis (8–12 weeks gestation) did not demonstrate any effect on testosterone production following exposure to 10 μmol/L for 24, 48, or 72 h [34▪▪]. One study has investigated the effect of exposure to a therapeutic regimen (60 mg/kg/d) of paracetamol (60 mg/kg/d) on human fetal testis (14–20 weeks gestation) xenografts. In this study 7 day exposure to paracetamol significantly reduced seminal vesicle (androgen dependent organ) weight (18% reduction) and serum testosterone (45% reduction) in the castrate nude mice hosts compared to vehicle controls, whilst a similar effect did not occur following a shorter (1 day) exposure [30▪▪]. Differences between the results of the two studies using human fetal testis may relate to the different gestational ages or alternatively may reflect differences in model system.

Similar studies have been performed using NSAIDs. In vivo studies have demonstrated a significant reduction in AGD (~20% reduction) in males exposed to aspirin (150 mg/kg/day) or indomethacin (1 mg/kg/day) from GD11 to 14 [35], whereas no reduction in AGD was found in similar studies involving exposure during the MPW in rats [21▪▪,35], although in one of these studies testosterone production was reduced in the case of aspirin [21▪▪] but not indomethacin [36]. However, in vitro rat fetal testis studies have demonstrated a reduction in testosterone production following 24 h exposure to aspirin (70% reduction; 10 μmol/L) and indomethacin (30% reduction; 10 μmol/L) from e14.5 [21▪▪,31]. These findings contrast with studies using in vitro culture of human testis [34▪▪]. Exposure of human fetal testis (8–12 GW) to indomethacin for 48 h resulted in a significant increase in testosterone (~20%; 10 μmol/L). A similar increase was demonstrated for aspirin exposure, although this was restricted to 8–10GW, with no effect at 10–12GW [34▪▪]. The reason for the discrepancy between the effect of NSAID exposure on testosterone production in the fetal rat and human testis is unclear and may relate to the model systems or to genuine species differences; however, this clearly illustrates potential limitations of extrapolating effects in rodent model systems directly into the human.

Analgesic exposure and Insl3 production

INSL3 is responsible for the first phase of testicular descent and mutations in INSL3 gene may lead to cryptorchidism in mice [37]. Paracetamol exposure (350 mg/kg/d) did not result in a change in *Insl3* mRNA in rat fetal testis following in-utero exposure from e13.5 to e16.5 [30▪▪]. This was also the case for Insl3 measured in the media following in vitro culture of rat fetal testis (e14.5) for 72 h [31]. However, in human fetal (8–12GW) testis cultures paracetamol (10 μmol/L; 72 h) exposure resulted in a significant reduction in Insl3 production, whilst no effect was observed following exposure to the same concentrations of aspirin or indomethacin [34▪▪].

Overall, the experimental studies suggest that exposure to analgesics can result in effects on Leydig cells in the fetal testis which may have the potential to result in male reproductive disorders. Whilst this conclusion is supported by the results of studies utilizing human fetal testis tissue there remain some important questions relating to the dose and duration of exposure and the degree of hormonal suppression that might be required to induce male reproductive disorders in humans. In addition, the mechanism by which analgesics might affect Leydig cell function requires further elucidation.

Effect of analgesics on prostaglandins

Prostaglandins have been proposed to play a role in mediating the effects of paracetamol exposure on Leydig cell function. Culture of e14.5 fetal rat testis showed a significant decrease in prostaglandin D2 (PGD2) after 24 h exposure to 1 μmol/L paracetamol [21▪▪]. However, there were no significant reductions across a range of doses (1–100 μmol/L) for 24, 48, and 72 h in a subsequent study by the same authors [31]. For human fetal testis (7–12 weeks) in vitro exposure to paracetamol (10 μmol/L) for 72 h did not reduce prostaglandin D2 (PGD2) production, but it did significantly reduce prostaglandin E2 (PGE2) [34▪▪].

For NSAIDS, the effect of exposure on prostaglandins appears to depend on the specific agent. A dose dependent reduction in PGD2 after exposure to aspirin was demonstrated in culture of e14.5 fetal rat testis after 48 and 72 h [21▪▪]; however, this was not confirmed in a subsequent study, with nonsignificant reductions only occurring at 100 μmol/L [31]. Similarly, for human fetal testis culture (7–12 weeks), no effect on PGD2 was observed following aspirin (10 μmol/L) exposure. However, similar to results for paracetamol there was a significant reduction in PGE2 following aspirin exposure [34▪▪]. For indomethacin, PGD2 was reduced following in vivo and in vitro exposure of the fetal rat testis during the MPW [21▪▪,36,38▪▪], which again was not demonstrated in human fetal testis cultures [34▪▪].

As with paracetamol and aspirin, exposure to indomethacin did result in a significant reduction in PGE2, albeit this was restricted to 7–10 weeks of gestation. Taken together, the present data relating to the effect of analgesics on prostaglandins appear to indicate variable effects on prostaglandins dependent, at least in part to the specific agents and the model species.

Effect of analgesics on germ cell development and fertility

Recent studies have begun to focus on the potential for analgesics to affect germ cell development and fertility including inter-generational effects [38▪▪,39▪▪]. Dean *et al.* investigated the effects of exposure of pregnant rats to 350 mg/kg/day paracetamol or 0.8 mg/kg/day indomethacin, during a period of gestation that includes the MPW. Pups exposed to indomethacin (male and female) showed ~50% decreased GC number and a decreased gonadal weight at e21.5 [38▪▪]. For females, this resulted in reduced fertility, as indicated by a reduced number of pups per litter, whereas in males no effect on fertility was seen. For paracetamol exposure, there was a similar effect on females with significant reduction in germ cell number, gonadal weight, and pups per litter [38▪▪], with effects on fertility also described for female mice exposed to paracetamol in-utero [39▪▪]. However, for males, despite an overall reduction in germ cell number and gonadal weight at e21.5, there was no significant effect on fertility [38▪▪]. Investigation of the reduced germ cell numbers in males revealed premature loss of gonocytes following exposure to both paracetamol and indomethacin. The loss of this proliferative population of germ cells is likely to result in the reduced germ cell number; however, this is compensated for by early puberty [38▪▪]. Another study involving paracetamol (50 mg/kg/d) exposure in mice (e7–e13.5) showed no effect on male germ cells at e13.5 or on germ cells or testicular weight in adulthood [32▪]. The differences between the findings in terms of the gonocyte population in fetal life may relate to differences in species, paracetamol dose, or timing of exposure.

Interestingly, recent studies have demonstrated effects of analgesic exposure on the F2 generation of rats exposed to paracetamol in utero. The F2 females exhibited a significant reduction in ovarian weight and in primordial follicle number at pnd25. Remarkably, this was seen independent of whether the F1 parent was male or female, raising the intriguing possibility that this may be as a result of epigenetic modification of the germline in both sexes [38▪▪].

These recent rodent studies demonstrate that analgesic exposure can affect germ cell development in the fetal testis; however, these findings need to be reproduced in human studies, including epidemiological and experimental approaches.

CONCLUSION

Over the past 5 years, several studies have investigated the potential effect of analgesic exposure to (paracetamol and NSAIDS) on the development of male reproductive disorders. Epidemiological evidence exists for associations between exposures to several analgesics and the development of cryptorchidism. Experimental studies in rodents have also demonstrated effects during fetal life on Leydig cell function (including testosterone production) and fertility. Recent in vitro and ex vivo (xenograft) studies using human fetal testicular tissue have lent support to the concept that analgesic exposure may interfere with Leydig cell function in the fetal testis. However, differences remain between the findings of these studies that are likely to reflect variations in species, model system, dosing schedule, and timing of exposure. Further work is required to determine the potential risk that analgesics may pose to human reproductive health at human-relevant exposures. Whilst the current evidence does not support a definitive answer to this question, avoiding pain or pyrexia is important for fetal health. With this in mind, a pragmatic approach would be to use analgesics only when necessary and for the shortest possible duration.

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Conflicts of interest

None.

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Analgesics affect fetal germ cell development in rodent and human

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Conflict of interest

The authors declare that they have no conflicts interests.

Abstract

In light of growing evidence that analgesic exposure during pregnancy can target the fetal gonads, including germ cells (GC) in rodents, we investigated whether therapeutically relevant doses of acetaminophen and ibuprofen affect GC development in the human fetal testis and ovary using in vitro (hanging drop culture) and xenograft model systems. We show that in fetal gonad culture the presence of analgesic reduces AP2γ^{+} (gonocyte) GC number in both 1st trimester fetal testes (22-28% reduction) and ovaries (43-49% reduction). Exposure of xenografted 2nd trimester fetal testes to 1 or 7 days acetaminophen led to reductions of 17% and 30%, respectively in AP2γ^{+} GC number. To determine mechanism of action, a human GC tumor-derived cell line (NT2) that exhibits fetal GC characteristics was used. This revealed that exposure to analgesics or prostaglandin E_2 (PGE_2) antagonists similarly reduced NT2 cell number, and PGE_2 agonists prevented acetaminophen-induced cell loss. Both analgesics and PGE_2 antagonists reduced expression of GC pluripotency genes and altered expression of key genes that regulate DNA and histone methylation in NT2 cells. Similar gene expression changes were induced in rat fetal testis/ovary cultures as well as in vivo after acetaminophen exposure of pregnant rats, demonstrating translatability of in vitro findings to in vivo and across species. Our results demonstrate robust GC/NT2 cell effects of analgesics, involving PGE_2 pathways, and raise concerns that similar effects might occur following analgesic use in human pregnancy, including potential next generation effects.

Introduction

Analgesics are used by the majority (55-80%) of pregnant women at some point during pregnancy *(1-3)*. This primarily involves use of 'mild' analgesics, such as acetaminophen (paracetamol) and ibuprofen (a non-steroidal anti-inflammatory drug; NSAID), which are available without medical prescription *(3, 4)*. Acetaminophen and NSAIDS are able to cross the placenta into the fetal circulation and as a result have the potential to affect fetal development *(5-8)*.

Epidemiological studies suggest that fetal exposure to analgesics has adverse effects on the male reproductive tract by consistently identifying an association between analgesic use during pregnancy and an increased risk of cryptorchidism in their sons *(1, 2, 9-11)*. Testicular descent is under the influence of testosterone produced by the Leydig cells of the fetal testis, and experimental studies have shown that the analgesics, acetaminophen, ibuprofen and aspirin all reduce testosterone production by the fetal testis in the rat *(10, 12, 13)*. More limited studies using human fetal testis tissue from relevant periods of gestation also demonstrate effects of acetaminophen exposure on hormone (including testosterone) production *(13, 14)*.

Suboptimal androgen production or action during fetal life is associated with the development of the common male reproductive disorders cryptorchidism and hypospadias, but also with risk of testicular germ cell cancer and effects on fertility *(15)*. Consequently, more recent studies have investigated analgesic exposure during pregnancy in relation to fetal germ cell (GC) development and later reproductive function in rodents *(16, 17)*. Modifications in fetal GC might not only affect the future reproductive potential of the exposed fetus, but can potentially be passed on to affect future generations. In experimental rat studies, gestational exposure to indomethacin or acetaminophen reduced fetal GC number by 40-50% in both sexes, with demonstrable consequences for adult fertility in females *(16)*. Moreover, it was shown that such fetal exposure to analgesics leads to abnormal ovarian development and function in the second generation, an inter-generational effect that was transmitted via both paternal and maternal lines *(16)*. It is hypothesized that such effects are indicative of epigenetic changes to the GC as a consequence of analgesic exposure in fetal life *(16)*, but this has not so far been investigated.

One potential mechanistic pathway that might link analgesics and epigenetic alterations is the prostaglandin (PG) pathway, through which acetaminophen, indomethacin (an NSAID) and ibuprofen all act. Somatic and germ cells in the fetal gonads of both sexes in rats and humans are sources and targets of PGs as they express the PG-synthesizing COX2 enzyme and PGE receptors *(16, 18)*. Numerous studies have shown that PGE_2 exerts a range of effects on normal and cancerous cells, in vivo and in vitro. These include alterations in cell proliferation *(19)* and stem cell pluripotency *(20, 21)*. Changes in epigenetic markers, such as DNA and histone methylation, are also described and reported to be mediated by PGE_2-induced changes in the expression of key epigenetic regulatory factors including DNA methyltransferases (DNMT3a and b) and enhancer of zeste homolog 2 (EZH2) *(22-24)*.

With this emerging background, the present study aimed to determine the effect of analgesic exposure, at therapeutically relevant levels, on GC number and pluripotency in the human fetal testis and ovary, to identify if this involves the PGE_2 pathway, and whether it results in changes in expression of key epigenetic regulatory factors. We show that exposure to either acetaminophen or ibuprofen can reduce GC number in human fetal gonads. Further analysis in NTERA-2 (NT2) cells, a human embryonal carcinoma (germ cell tumour) cell-line, showed a reduced cell number after analgesics exposure and PGE_2 receptor antagonists. Moreover, experiments on NT2 cells and in rats, both in vitro and in vivo, demonstrate consistent modifications in expression of genes involved in GC pluripotency and epigenetic regulation. We identify robust and species-independent GC effects of analgesics, which raise the possibility of similar effects in human pregnancy.

Results

The effect of common analgesics was studied in a variety of model systems in vitro and in vivo. The models and their specific treatment regimens are shown in Figure 1.

Effects of 7-day acetaminophen or ibuprofen exposure on GC in hanging drop cultures of 1st trimester human fetal gonads

Analgesic effects during pregnancy may depend on the gestational period *(9, 10)*, so we investigated effects in both 1st and 2nd trimester fetal gonads. The effect of acetaminophen or ibuprofen exposure on GC during the 1st trimester was investigated using a hanging drop model *(25)*, in which testis and ovary samples were exposed to vehicle or analgesic (acetaminophen or ibuprofen; both 10μM) for 7 days (Figure 1A). Cultured tissue pieces appeared healthy. Total GC number and the ratio of gonocytes to pre-spermatogonial subpopulations were calculated using activating enhancer-binding protein 2 (AP2γ or *TFAP2C*; gonocytes) and melanoma-associated antigen 4 (MAGE-A4; pre-spermatogonia) as GC markers, and sex determining region Y-box 9 (SOX9) to mark Sertoli cells (Figure 2A,B and C).

Acetaminophen exposure significantly reduced total GC number (AP2γ^+ + MAGE-A4$^+$) and the number of gonocytes (AP2γ^+) by 18% (P<0.001) and 28% (P<0.01) respectively (Figure 2D and E). Although ibuprofen similarly reduced total GC number and AP2γ^+ GC, only the reduction in AP2γ^+ GC was statistically significant (22% reduction, P<0.01; Figure 2E). Presence of MAGE-A4$^+$ GC was so infrequent in 1st trimester fetal testes that their numbers were insufficient to enable meaningful statistical analysis. Apoptosis was studied using and Cleaved caspase 3, but apoptotic cells were rarely identified across all treatments and therefore, quantification was not performed (Supplemental figure 1).

As the reduced germ cell number in analgesic-exposed samples could not be explained by an increase in apoptosis, we used Ki-67 co-immunostaining (Figure 2F) to investigate whether altered GC proliferation was a possible explanation. Exposure to either analgesic resulted in a reduction in the proportion of proliferative AP2γ^+ gonocytes with a similar level of reduction for acetaminophen (22%, P<0.05) and ibuprofen (28%, P<0.05) (Figure 2G).

For fetal ovary analyses, we followed a similar approach, staining AP2γ^+ GC and using Hoechst as a counterstain (Figure 3A,B and C); MAGE-A4 staining was not included since it is not a marker of ovarian GC differentiation. The number of AP2γ^+GC was significantly

reduced (Figure 3D) by exposure to either acetaminophen (43% reduction; $P<0.05$) or ibuprofen (49% reduction; $P<0.01$). Ovarian AP2γ^+GC proliferation was calculated in the same way as for fetal testes and revealed significant reductions in GC proliferation after culture with acetaminophen (25% reduction, $P<0.01$) or ibuprofen (35% reduction, $P<0.01$) (Figure 3E).

Effect of 7-day acetaminophen or ibuprofen exposure on GC in 2nd trimester human fetal testis xenografts

Second trimester testis tissue samples were xenografted into nude mice as described previously *(13)*. Treatment of host mice with analgesics for 7 days (Figure 1B) led to a reduction in total GC number (AP2γ^+ + MAGE-A4$^+$) for acetaminophen (38%; $P<0.05$; Figure 4A) and ibuprofen (53%; $P<0.05$; Figure 4E), respectively, in comparison with vehicle–treated hosts. In acetaminophen-exposed samples, this reduction appeared to result mainly from a decrease in the AP2γ^+ GC subpopulation (30% reduction, $P<0.05$; Figure 4B), and although a similar trend was evident for ibuprofen-exposed samples this was not statistically significant (Figure 4F). The MAGE-A4$^+$GC subpopulation was not significantly modified by either of the analgesic treatments (Supplemental figure 2). Proliferation of total GC and AP2γ^+ GC did not show a significant reduction after acetaminophen or ibuprofen exposure (Supplemental figure 3).

Effect of 1-day acetaminophen exposure on GC in 2nd trimester human fetal testis xenografts

As acetaminophen usage in pregnancy is likely to be for short periods (≤24h), we investigated whether 1-day exposure to acetaminophen, followed by 6 days of vehicle treatment would affect GC number and proliferation in the xenograft model (Figure 1B). This exposure led to a significant reduction in total GC (22%; $P<0.05$) and AP2γ^+ GC number (17%; $P<0.05$) in xenografts (Figure 4C and D), although the reduction was less pronounced compared to 7-day acetaminophen exposure (Figure 4A and B). MAGE-A4$^+$GC number was not significantly altered by 1-day acetaminophen exposure (Supplemental figure 2). GC proliferation (total or AP2γ^+) showed a trend towards a reduction after 1 day exposure to acetaminophen, similar to that seen with 7 day exposure to acetaminophen and ibuprofen, but this was not statistically significant (Supplemental figure S3).

Effects of acetaminophen, ibuprofen and EP2+EP4 antagonists on NT2 cell number

In view of the effects of analgesic exposure on GC number in human fetal testis tissue, we utilized a more tractable system to dissect possible mechanisms of analgesic action. We chose NT2 cells, a pluripotent human embryonal carcinoma cell line derived from an adult testicular GC cancer patient. Due to the fetal origin of testicular GC cancer, NT2 cells express markers of fetal GC, such as AP2γ and hence they are commonly used as a fetal GC model *(26)*.

Ibuprofen/NSAIDs and acetaminophen can act via PG signaling pathways *(27-29)*, including in the fetal rat ovary in which we have previously described effects of analgesic exposure on fetal GC number *(16)*. Therefore, in addition to investigating the effects of acetaminophen and ibuprofen on NT2 cells, we also investigated whether blockade of PGE_2 action via combined exposure to an EP2 (PF-04418948, 10μM) and an EP4 (L-161,982, 10μM) receptor antagonist, could induce effects similar to those of acetaminophen and ibuprofen. NT2 cells were exposed for 48h to different doses of acetaminophen (10-50μM), ibuprofen (10μM) or EP2+EP4 antagonists (10μM each; Figure 1C). All three types of exposures significantly reduced the number of NT2 cells after 48h (Figure 5A-C) suggesting that the analgesic-induced decrease in NT2 cell number could involve inhibition of the PGE_2 pathway. To test this, we cultured NT2 cells either with acetaminophen (10μM), with EP2+EP4 agonists (Butaprost 10μM + CAY10598 10nM) or with the two treatments combined (Figure 1C). Exposure to the EP2+EP4 agonists alone had no significant effect on NT2 cell number, whereas exposure to acetaminophen significantly reduced ($P<0.05$) cell number (Figure 5D). The effect of acetaminophen was completely prevented by co-culture with the EP2+EP4 agonists, demonstrating that acetaminophen-induced NT2 cell loss involves an intact PGE signaling pathway (Figure 5D).

Effects of acetaminophen, ibuprofen or EP2+EP4 antagonists on expression of GC differentiation markers in NT2 cells

A previous study has shown that analgesic exposure of rats during gestation resulted in premature loss of expression of the gonocyte pluripotency marker octamer-binding transcription factor 4 (OCT4 or POU5F1) in the fetal testis *(16)*. We therefore investigated whether in-vitro culture of NT2 cells with acetaminophen, ibuprofen or EP2+EP4 antagonists altered expression of the established GC pluripotency markers *OCT4*, *AP2*γ and *NANOG (30)*. Acetaminophen exposure significantly reduced expression of all three genes in NT2 cells (Figure 6A), while ibuprofen caused a significant reduction only in *OCT4* expression (Figure 6C). Culture of NT2 cells with EP2+EP4 antagonists also significantly reduced expression of

OCT4, *AP2γ* and *NANOG* (Figure 6E) to a similar degree to that seen with acetaminophen exposure.

Effects of acetaminophen, ibuprofen or EP2+EP4 antagonists on expression of epigenetic regulatory genes in NT2 cells

Following previous studies showing inter-generational consequences of in utero acetaminophen/NSAID exposure in rats *(16)* and other evidence that PGE_2 may fundamentally regulate the epigenetic machinery *(22, 24)*, we hypothesized that exposure to acetaminophen, ibuprofen or EP2+EP4 antagonists might exert similar effects on epigenetic regulatory genes in NT2 cells. We focused on DNA and histone methylation. Thus, we studied expression of the DNA methyltransferases *DNMT3a* and *DNMT3b*, Ten-eleven translocation 1 (*TET1),* which plays a role in DNA and histone methylation, and *EZH2* which is the enzymatic component of the polycomb repressive group 2 (PRC2) complex, in charge of adding the repressive H3K27me3 histone methylation mark.

DNMT3a expression by NT2 cells was decreased by ibuprofen and unaffected by either acetaminophen, or EP2+EP4 antagonists, whereas *DNMT3b* expression was reduced after culture with acetaminophen or EP2+EP4 antagonists, but not by ibuprofen (Figure 6B,D and F). Conversely, expression of *TET1* was increased when NT2 cells were cultured with acetaminophen, ibuprofen or EP2+EP4 antagonists, while *EZH2* expression was only increased significantly by EP2+EP4 antagonists (Figure 6B,D and F). However, the relative amount of H3K27me3, as measured by ELISA, was significantly increased after culture of NT2 cells with acetaminophen, ibuprofen or EP2+EP4 antagonists (Figure 6G).

In vivo and in vitro effects of acetaminophen, ibuprofen or EP2+EP4 antagonists on fetal rat gonads

Having established that PGE_2 is involved in mediating effects of analgesic on NT2 cell number and gene expression (and thus likely also for effects on GC number in human fetal gonads), we next investigated whether similar gene expression changes could be induced in vivo. As it is not possible to perform in-vivo human studies, we performed these studies in the rat, as we have already shown that analgesic exposure in pregnancy will reduce fetal GC numbers in both sexes *(16)*. Initially, we confirmed the findings relating to the epigenetic regulatory genes using fetal rat ovarian or testicular tissue in hanging drop culture. Tissue pieces were cultured for 48h with or without exposure to acetaminophen (10μM), ibuprofen (10μM) or EP2+EP4

antagonists (10μM L-161,982 + 10μM PF04418948; Figure 1D). In fetal testes and ovaries, *Tet1* expression was increased by all treatments while *Ezh2* expression was significantly increased by acetaminophen and ibuprofen but not by EP2+EP4 antagonists in both testis and ovary cultures (Figure 7A-F), similar to that described for NT2 cells for *TET1* (all treatments) and *EZH2* (EP2/EP4 antagonists). Expression of *Dnmt3b* was significantly reduced by exposure to EP2+EP4 antagonists in both testis and ovary cultures (Figure 7C and F) whereas expression of *Dnmt3a* was only reduced in ovary cultures (Figure 7F);

To provide in vivo evidence for the relevance of the identified gene expression changes induced by analgesics, we used pregnant rats, which were administered 350mg/kg acetaminophen (a human equivalent dose of ~60mg/kg based on calculation of body surface area) *(31)* by gavage once daily from e13.5 until the day before sampling (either e15.5 or e17.5) (Figure 1E), as described previously *(16)*;. Similar to the in vitro culture studies, acetaminophen exposure in vivo significantly increased expression of *Tet1* in both the fetal testis (e15.5 and e17.5; Figure 8A and B) and ovary (e15.5 only; Figure 8C and D), while *Ezh2* expression was significantly increased only in e17.5 testes (Figure 8B) and e15.5 ovaries (Figure 8C). Acetaminophen exposure significantly reduced expression of *Dnmt3a*, but not *Dnmt3b*, in e15.5 ovaries (Figure 8C), consistent with in vitro findings already described (Figure 7F).

Discussion

Our results show that exposure to the analgesics acetaminophen and ibuprofen, used commonly by pregnant women, causes significant reductions in GC number in both human fetal testes and ovaries under our experimental test conditions, as well as altering expression of GC-differentiation markers and epigenetic regulatory genes in a consistent manner in NT2 cells, a surrogate for fetal GC. The analgesic-induced reduction in GC number in the fetal testis was evident across the gestational range examined and could be induced even with a 1-day treatment (acetaminophen only) at a human-relevant therapeutic dose. These results are important because the majority of women take at least one analgesic during pregnancy and previous studies in pregnant rats have shown that analgesic exposure can affect the future fertility of newborn females *(16, 17)*. Our results also provide robust evidence that these effects of analgesics are likely to involve, or be mediated through the PGE_2 pathway, and that one consequence of affecting this pathway is altered expression of a number of genes that play key roles in epigenetic regulation. This raises the possibility that analgesic exposure might

alter the epigenome of fetal GC, which could potentially affect the next generation, as previously demonstrated by in vivo studies in the rat *(16)*.

As direct translation of our earlier findings of adverse effects of analgesic exposure on fetal GC in the rat *(16)* to the human is not possible, we employed in vitro and ex vivo (xenograft) model systems using fetal testicular or ovarian tissue or NT2 cells. Despite their respective limitations, exposure to either acetaminophen or ibuprofen at human-relevant concentrations reduced GC/NT2 cell number in all of these systems. These findings are similar to those of previous studies in the rat and mouse that showed a decrease in GC number after analgesic exposure during pregnancy *(16, 17)*. In the human fetal testis, in culture (1st and 2nd trimester) or in xenografts (2nd trimester) the analgesic-induced reduction in GC number was most apparent for the AP2γ^+ gonocytes compared to the more differentiated pre-spermatogonia (MAGE-A4$^+$), raising the possibility that the analgesic effect is targeted mainly to a specific fetal GC subpopulation. The AP2γ^+ GC in the human fetal testis are highly proliferative *(32)*, and in keeping with this it appeared that analgesics most likely induced GC loss via reduced proliferation, although this is a tentative conclusion. In fetal human ovary cultures, the highly proliferative premeiotic oogonia (AP2γ^+) were similarly reduced in number following analgesic exposure. Analgesic targeting of AP2γ^+ GC may be related to the probable mechanism of analgesic action via PGE_2, as discussed below, as the PGE_2 pathway appears to be important in modulating proliferation of (pluripotent) stem cells in various other systems *(19, 23, 33, 34)*.

In the xenograft model, which has been shown to recapitulate normal human fetal testis GC development *(32)*, AP2γ^+ GC number was significantly reduced by a single day's exposure to acetaminophen using a human-relevant exposure regimen. Similarly, in the in vitro human fetal gonad cultures, reduction in GC number by analgesics occurred at concentrations in the lower range of levels reported in blood after normal human usage of acetaminophen or ibuprofen *(14, 35, 36)*. Moreover, our findings that analgesic exposure induced similar levels of GC loss in the human fetal testis and ovary in each model system used, as well as causing similar GC loss in rats in fetal gonads after analgesic exposure in vivo *(16)*, demonstrates the robust nature of the effect. This raises the possibility that analgesic use in human pregnancy will result in similar GC loss. The potential consequences of analgesic-induced fetal GC reduction in humans are unknown. In rats, fetal GC reduction in males can be completely compensated postnatally as the differentiated GC resume proliferation after birth *(16)*, and it

is likely that this would also occur in human males. However, proliferation of GC in the human female is restricted by subsequent entry to meiosis, which occurs from 9 weeks gestation *(37, 38)*, so that any GC loss during or after this period could have long-term consequences for oocyte reserves and reproductive lifespan *(39)*. In keeping with this, previous studies in pregnant rats showed that analgesic-induced decrease in fetal GC number translated into a decrease in adult ovarian weight and reduced fertility as indicated by a reduction in pups per litter after mating *(16)*. This concern might be possible to address in humans by relating analgesic use in pregnancy to blood levels of anti-müllerian hormone (AMH) in resulting female offspring, as AMH levels are related to oocyte reserve under certain conditions *(40)*.

An important aim of our experiments was to establish a likely mechanism for the analgesic effects on fetal GC. Both analgesics have well established effects on prostaglandin pathways *(27-29)*. Our in vivo rat studies had shown that analgesic exposure reduced PGE_2 levels in the fetal gonads *(16)* and that fetal GC in both the rat *(16)* and human *(18)* are PGE_2 targets, so we focused on this pathway. As isolation and culture of human fetal GC was not feasible, we decided to use a more tractable model for fetal human GC, NT2 cells. These cells represent a human pluripotent embryonal carcinoma (EC) cell line that expresses markers (e.g. AP2γ, OCT4, NANOG) similar to undifferentiated (pluripotent) human fetal GC *(41)*. The present data show that analgesic effects on NT2 cell number and pluripotency gene expression are mimicked by culturing with EP2+EP4 receptor antagonists (which block PGE_2 action), and that the acetaminophen-induced decrease in NT2 cell number can be prevented by concomitant exposure to EP2+EP4 agonists. This demonstrates that analgesic effects on NT2 cells are dependent on a functional PGE_2 pathway. This is further reinforced by the finding that PGE_2 antagonism induced similar gene expression changes to those induced by analgesics in both human (NT2 cells) and rat (fetal gonad) systems.

Our studies with NT2 cells suggest that one effect of analgesic exposure or PGE_2 antagonism is to reduce expression of GC pluripotency genes, such as *OCT4*, *AP2*γ and *NANOG*. This is consistent with previous studies showing that exposure of mouse embryonic stem cells to an EP2 antagonist led to down-regulation of pluripotency genes, such as *Oct4* and *Nanog (42)*, and abundant other evidence for a fundamental role for PGE_2 in regulating both stem cell and cancer cell proliferation and differentiation status in numerous cell types *(23, 34, 43, 44)*. Moreover, experiments exposing human fetal testis explants to ibuprofen also found a reduction in specific germ cell genes involved in pluripotency including *OCT4* and *LIN28A*

(45). In a human context, arrested differentiation of fetal GC is now accepted as being the primary pathway to development of testicular GC cancer (TGCC), the commonest malignancy amongst young men *(46)*. However, analgesic-induced reduction in expression of pluripotency genes in NT2 cells, as well as in vitro (this study) and in vivo in the rat fetal testis *(16)*, appears to be in the opposite direction to what is thought to underlie the origin of TGCC.

Our demonstration that analgesic exposure induces robust changes in expression of epigenetic regulatory genes in NT2 cells as well as in fetal ovaries and testes from the rat (in vitro and in vivo) could be a consequence of selective loss of a specific cell subtype in the test systems, for example loss of pluripotent GC in fetal rat gonads. An alternative possibility is that the analgesic-induced changes in expression of epigenetic regulatory genes could alter the epigenome in exposed GC with unknown consequences. This would be consistent with our finding that fetal exposure of rats to analgesics results in inter-generational transmission of effects via both paternal and maternal lines *(16)*. Our present demonstration that total H3K27me3 was significantly increased in NT2 cells by acetaminophen, ibuprofen and EP2/EP4 antagonists, could suggest that alterations in this repressive histone mark in specific genes has occurred, and is less easily reconciled with selective loss of a particular GC population. In this respect, H3K27me3 is highly expressed and dynamically regulated in fetal GC and is thought to be an important factor in fetal GC differentiation and development *(47, 48)*, although our studies did not delineate which genes might have been affected by the global change in H3K27me3. We also showed that analgesics and PGE_2 antagonism decreased expression of the key methyltransferase enzymes DNMT3a and DNMT3b, in agreement with previous studies linking PGE_2 action with DNA methylation *(22, 23, 49)*. As fetal GC undergo extensive de- and then re-methylation during fetal or early postnatal development *(48, 50)*, analgesic exposure could potentially impact this process via their effects on *DNMT* expression.

Whilst our findings can only be viewed as circumstantial support for the possibility that analgesics might induce epigenetic changes in fetal GC in human pregnancy, our different model systems provide the means via which this can be investigated more directly than at present. What seems clear from our present and previous results is that PGs, and specifically PGE_2, appear to play a fundamental and conserved role in modulating key components of the cellular epigenetic regulatory machinery in the fetal gonads, as is the case in other tissues *(22, 24)*. This raises the possibility that analgesic use/exposure might have other effects (beneficial

or adverse), besides their current use and that these could be targeted for additional therapeutic purposes.

Our study has limitations. While none of the models can accurately recreate in vivo conditions for humans, the xenograft and culture models have been shown to recapitulate key aspects of normal human fetal development, including GC proliferation and differentiation *(25, 32, 51)* and may provide some insight into *in utero* exposure. Our use of NT2 cells also has limitations, as it is not a true fetal GC line. However, NT2 cells derive from TGCC precursor cells, which are believed to originate from fetal GC *(26)*. Despite these limitations, the consistency of GC/NT2 cell effects and the concordance of gonadal tissue GC/NT2 cell and gene expression changes between humans and rats, including in vivo in the latter, suggest that the mechanisms involved in analgesic/PGE_2 effects are highly conserved and therefore likely to operate in vivo in humans.

In conclusion, the present data show that exposure of human fetal ovaries or testes to therapeutically-relevant concentrations of acetaminophen and ibuprofen, which are commonly used by pregnant women, causes a consistent decrease in fetal GC number as well as inducing changes in gene expression and, potentially, epigenetic modifications. These effects are robust, conserved in rat and human across different model systems, and are likely to result from disruption of PGE_2 pathways. Although translation of these results to human pregnancy has to be considered with caution, the robust nature of our findings and the apparent involvement of a conserved mechanism, support recommendations that analgesic use in human pregnancy should only be used if needed, and then only for the shortest period necessary to manage symptoms. Our present findings, taken together with a growing body of evidence for other effects of analgesics during pregnancy on a range of human developmental outcomes *(10, 13, 14, 52, 53)*, and the high prevalence of analgesic use during pregnancy, suggest that properly designed prospective studies to investigate potential associations between analgesic use and outcomes in offspring, should be a research priority.

Materials and Methods

Experimental design

Given the reported association between maternal use of common analgesics and effects on fetal GCs and fertility, we aimed to determine whether exposure to acetaminophen or ibuprofen affects GC number and to further examine the mechanisms involved. Effects of exposure were studied in human fetal gonads taken from different stages of pregnancy using hanging drop culture (1st trimester testes and ovaries) and a xenograft system (2nd trimester testes). Samples were exposed to analgesics in the lower range of human-equivalent therapeutic exposure. Experiments to determine mechanism of analgesic action on GC were conducted in a GC-tumor derived cell line (NT2) and end-points evaluated included cell number, proliferation status and expression of pluripotency markers and epigenetic regulators. Further in vitro and in vivo studies in rats allowed us to demonstrate similar analgesic effects and mechanism as in human and translatability of our findings from in vitro to in vivo (pregnancy). Inclusion criteria and measured endpoints were defined before the start of the study. For human fetal gonad experiments, the sample sizes (minimum n=3) were based on those required to achieve statistical significance in previous studies using the same methodology *(54, 55)*. The study was stopped once the required number of experiments had been conducted, and data were analyzed after the cessation of the study. For fluorescence immunohistochemistry, all samples from the same experiment were stained in the same run. GC/NT2 number was determined blind to the treatment group. To compare the effects of treatment versus vehicle in xenografts for each individual human fetal testis, we grafted tissue from each fetus into three to six replicate host mice and randomly allocated these to receive either acetaminophen/ibuprofen or vehicle treatment. For the rat studies, pregnant dams were randomly allocated to receive either acetaminophen or vehicle via oral administration.

Animals

All aspects of animal housing, management and treatment conformed to UK Home Office guidelines. Wistar rats were housed under standard conditions and nude mice were housed in IVCs. All animals had free access to tap water and soy-free diet (SDS; Dundee, Scotland). For rat studies, timed-matings were established by the presence of a vaginal plug, defined as embryonic day 0.5 (e0.5).

Human fetal gonad ‘hanging drop’ cultures

Culture of 1st trimester human fetal testes (n=4; 8-11 gestational weeks) and ovaries (n=3 9-11 gestational weeks) were cultured based on previous tissue cultures studies *(12, 14, 25)*. Gonads were dissected and cut into ~1mm^3 pieces. Single pieces were cultured in hanging drops containing 30µL culture medium (Alpha-MEM; Lonza) supplemented with 10% fetal bovine serum (Life Technologies), 10µL/mL penicillin/streptomycin (Sigma-Aldrich), 1% 200mM L-glutamine (100X) (Gibco), 1% MEM NEAA non-essential amino acids (100X) (Gibco), 2% sodium pyruvate 100mM (100X) (Gibco) and 1% Insulin-transferrin-Selenium (100x) (Gibco). Plates were incubated for 8 days at 37ºC under 5% CO_2. The medium was changed every day. Samples were cultured with hCG alone during the first day, and thereafter the medium was supplemented with hCG plus vehicle (DMSO) or analgesic (10µM acetaminophen or 10µM ibuprofen) for the remainder of the culture period (Figure 1). hCG (0.1U/ml; Pregnyl, Organon Laboratories) was added in order to mimic the in-utero environment. The analgesic concentrations are in the lower range of serum concentrations reported in humans following therapeutic analgesic exposure *(12, 14, 36)*. At the end of the culture period, tissue samples were fixed in Bouins (Clin-Tech) for 2h for subsequent analysis by fluorescence immunohistochemistry.

Human fetal testis xenograft studies

Second trimester human fetal testis tissue was used for ex-vivo xenografts studies, which provides a more physiological model to study gonad development *(32)*. Second trimester samples are larger than 1st trimester, and thus provide sufficient testis tissue to perform the xenografts. Previous attempts by our lab using human fetal ovarian tissue xenografts did not achieve good viability, therefore xenograft experiments were only performed with testis tissue. Fetal testes (n= 8; 14-17 gestational weeks) were cut into ~1mm^3 pieces and xenografted into castrate nude mice as described previously *(13)*. Male CD1 nude (host) mice (aged 4-6 weeks; n= 44; Charles River UK) were used for the xenograft studies. Mice were anesthetized by isoflurane inhalation and castrated through a scrotal incision at least 2 weeks before the xenograft procedure. Analgesia (Carprofen, Pfizer) was given in the drinking water for 3 days after castration. Four to six pieces of testis tissue were grafted subcutaneously under the dorsal skin of each host mice using a 13-gauge cancer implant needle (Popper and Sons); grafts were placed on either side of the dorsal midline. Grafts were maintained in the host for 7 days, in order to allow them to establish a blood supply, followed by 1 or 7 days of analgesic exposure (Figure 1B). Host mice also received subcutaneous injections of 20 IU hCG every 72h in order to mimic the in-utero environment *(13)*. Mice were randomly allocated to receive

vehicle or analgesic for either 1-day (followed by 6 days of no treatment) or 7-days. Host mice received vehicle (corn oil), acetaminophen (20mg/kg three times daily; Sigma-Aldrich) or ibuprofen (10mg/kg three times daily; Sigma-Aldrich); both analgesics were suspended in corn oil and administered by gavage. Host mice were sacrificed by cervical dislocation and grafts were retrieved and weighed before fixation in Bouins. Sections of this fixed tissue were subsequently used for fluorescence immunohistochemistry.

Cell culture

NT2 cells were used to study potential mechanisms of analgesic action on GC. For expansion, cells were cultured in DMEM media (Life Technologies), supplemented with 10% fetal calf serum (Life Technologies), 1% penicillin/streptomycin (Sigma-Aldrich) and 1% L-glutamine (Gibco). Cell cultures were washed with 1x Phosphate-buffered saline (PBS) (Life technologies), and disaggregated with TRypLE™ Express (Gibco). Cells were cultured at 37ºC under 5% CO_2 and split 1:2 or 1:4 for passage when confluent. Cells (200,000/well) were cultured in 6-well plates (Corning) for 48h at 37ºC under 5% CO_2. Media was supplemented with either vehicle (DMSO), 10 or 50μM acetaminophen, 10μM ibuprofen or 10μM L-161,982 (EP2 antagonist) + 10μM PF04418948 (EP4 antagonist) (Figure 1C).

For analysis with EP2+EP4 agonists, NT2 cells (excluding vehicle) were first exposed to 10μM Butaprost (EP2 agonist; Abcam) and 10nM CAY10598 (EP4 agonist; Abcam) for 4h before the medium was changed to the subsequent treatment: 10μM Butaprost + 10nM CAY10598, 10μM acetaminophen or the combination of acetaminophen + EP2/EP4 agonists (Figure 1C). The total length of exposure was 48h. After treatment, cells were either disaggregated with TRypLE for cell counting or protein analysis (ELISA), or collected for gene expression with Trizol (Life Technologies). All experiments were run in triplicate (n=3-4). Cell number was determined using a NucleoCounter (Chemometec) according to the manufacturer's guidelines. Disaggregated cells were re-suspended in medium and treated with lysis and stabilization buffer (Chemometec). Cells were counted using a NucleoCassete (Chemometec) on a NucleoCounter machine (Chemometec). Cell death was determined at the end of the experiment by flow cytometry (Supplemental figure 4). Propidium iodide (75μM; BD biosciences) was added to the disaggregated cells to identify the proportion of dead cells which was quantified by flow cytometry using a Flow Analyser 5L LSR Fortessa (BD).

Rat fetal gonad 'hanging drop' cultures

Rat fetal (e15.5) testes and ovaries were dissected and cut into ~1mm^3 pieces (n=13-30). Each piece was cultured as described for human hanging drop cultures. Tissues were cultured for 48h in either vehicle, acetaminophen (10μM), ibuprofen (10μM) or L-161,982 (EP2 antagonist; 10μM) + PF0441848 (EP4 antagonist; 10μM) (Figure 1D). Samples were then collected for fixation in Bouins or snap-frozen for gene expression analysis.

Rat fetal in vivo studies.

Pregnant female rats (n= 21) were administered acetaminophen (350 mg/kg/day by oral gavage) suspended in corn oil from e13.5 until the day before sampling (e15.5 or e17.5), as described previously *(15, 16)* (Figure 1E). This dose equates to a human equivalent dose of ~60mg/kg (i.e. typical daily consumption in humans) based on calculation of body surface area *(31)*, and has been shown to be non-toxic *(13)*. Control dams were administered corn oil according to the same regimen. After treatment, rats were sacrificed by cervical dislocation and fetal gonads were dissected and frozen for gene expression analysis.

Fluorescence immunohistochemistry

To identify specific testicular and ovarian cell populations, fluorescence immunostaining was used to identify Sertoli cells (SOX9; Merck, Millipore), pre-granulosa cells (FOXL2; Abcam) and GC subpopulations, namely gonocytes (AP2γ; Santa Cruz Biotechology) and pre-spermatogonia (MAGE-A4; Gift from Giulio Spagnoli). Proliferative GC were identified by co-immunostaining for Ki-67 (Abcam). Apoptosis was studied using Cleaved caspase (Cell signalling technologies) Sections (5μm) were mounted onto coated slides (BDH Chemicals), dewaxed in xylene and rehydrated using graded ethanol series. Sections were then subjected to antigen retrieval by pressure-cooking in 0.01M citrate buffer (pH 6.0) for 30 seconds at 125ºC, and sections remained in the decloaking chamber for a further 20 minutes after it cooled to 90ºC. Samples were then incubated in 3% (vol/vol) hydrogen peroxide (VWR chemicals) in methanol (Fisher Chemical) to block endogenous peroxidase activity. Sections were then washed with tris-buffered saline [TBS: 0.05 m Tris, 0.85% NaCl (pH 7.4)] and incubated for 30 min with 20% chicken serum, 5% bovine serum albumin (BSA) (Sigma) in TBS to block non-specific binding of the antibodies. Slides were then incubated with primary antibody, diluted in TBS, in a humidified chamber overnight at 4ºC. This step was followed by incubation with the appropriate secondary antibody IgG conjugated to horseradish peroxidase (HRP; Santa Cruz Biotechnology), at a concentration of 1:200 in TBS for 30 min at room

temperature. Sections were then incubated for 10 min with Tyramide (TSA Plus Cyanine 3 System, PerkinElmer Life Sciences) according to the manufacturer's instructions.

For double/triple immunohistochemistry, for each subsequent primary antibody, sections underwent further antigen retrieval by boiling in 0.01M citrate buffer (pH 6.0) for 2 min in a decloaking chamber. This was followed by blocking, overnight incubation with the primary antibody, incubation with the appropriate secondary antibody and Tyramide as described above. In some cases, this step was followed by a final incubation with the nuclear counterstain, Hoechst (Thermo Fisher), diluted 1:4000 in TBS for 10 min. Slides were washed in TBS and mounted in aqueous mounting medium (Permafluor; Beckman Coulter, High Wycombe). Images were acquired using a Provis microscope (Olympus Optical) fitted with a DCS330 digital camera (Eastman Kodak).

Quantification of GC number and GC proliferation in human fetal gonads

As only small pieces of fetal testicular and ovarian tissue were used for culture, analysis of GC number had to take account of tissue section area (ovaries) or seminiferous cord area (1st trimester testes), as this varied between samples, as previously described *(16)*. Seminiferous cord area was defined by the delineated structures containing Sertoli cells (SOX9$^+$). For 2nd trimester fetal testes we expressed GC number relative to Sertoli cell number, the main somatic cell component of the seminiferous cords, as previously described *(13)*. Thus, fetal testis sections (1 per cultured or xenografted tissue piece) were co-immunostained using antibodies for SOX9 (Sertoli), AP2γ (gonocytes) and MAGE-A4 (pre-spermatogonia). The number of cells from the different cell populations was counted manually and the area was determined (using ZEN software) by a single observer who was blinded to treatment. The total number of AP2γ^+ and MAGE-A4$^+$ GC per section was expressed relative to the total area of the seminiferous cords in that section. Three different populations were quantified: Total GC (AP2γ^+ + MAGE-A4$^+$), AP2γ^+ GC and MAGE-A4$^+$GC per μm^2. For 2nd trimester fetal testes, the number of GC was expressed relative to the total number of Sertoli cells (SOX9$^+$) in each section as previous studies have shown that Sertoli cell number in human fetal testis is unaffected by exposure to ibuprofen *(45)*. Three ratios were determined: Total GC (AP2γ^+ + MAGE-A4$^+$) per Sertoli cell, AP2γ^+ GC per Sertoli cell and MAGE-A4$^+$GC per Sertoli cell. For quantification of GC proliferation, sections were immunostained for AP2γ, MAGE-A4 and Ki-67. Proliferative GC (Ki67$^+$) were quantified for total GC (AP2γ^+ or MAGE-A4$^+$), AP2γ^+ GC and MAGE-A4$^+$GC populations as described above.

For the fetal ovary analysis, sections (1 per cultured piece) were co-immunostained using an AP2γ antibody (to identify all the GC) and Hoechst as a counterstain. The total number of AP2γ^+ GC per section was quantified and expressed relative to the total tissue section area. The number of AP2γ^+ GC per μm^2 was calculated and compared between vehicle and treatments. Proliferative AP2γ^+ GC (Ki67$^+$) were quantified as described for fetal testes.

Gene expression analysis

RNA extraction was performed using the RNeasy® Micro Kit (Qiagen) according to the manufacturer's instructions. For hanging drop cultures, all tissue pieces from the same gonad were pooled. For in-vivo rat studies, both ovaries from the same fetus were pooled, whilst a single testis from each fetus was ample to yield a sufficient quantity of cDNA. The RNA phase of the Trizol was separated and converted into cDNA using a Vilo kit (Invitrogen) according to the manufacturer's instructions. Gene expression analysis from the cDNA was performed using the Applied Biosystems Taqman™ RT kit (Applied Biosystems). Quantitative real time PCR (qRT-PCR) was performed using the ABI Prism Sequence Detection System (Applied Biosystems). Expression of human (*TET1*, *EZH2, DNMT3a*, *DNMT3b*, *OCT4*, *AP2*γ and *NANOG)* and rat (*Tet1*, *Ezh2*, *Dnmt3a* and *Dnmt3b)* genes were determined using the Roche Universal Probe Library (Roche Applied Sciences, Burgess Hill, UK). The ribosomal 18s internal control (Applied Biosystems) and the transcription factor TATA-binding protein (*TBP)* (NT2 cell analysis only) were used as reference genes to correct the expression of the gene of interest. All samples were analyzed in duplicate. Human primers: *TET1* forward primer: 5'-gatgacagaggttcttgcacat-3', reverse primer: 5'-aggttgcacggtctcagtgt-3', probe number #86 (cat. no. 04689119001); *EZH2* forward primer: 5'-aagaagagaagaaagatgaaacttcg-3', reverse primer: 5'-ttggtgtttgacaccgagaat-3', probe number #82 (cat. no. 04689054001); *DNMT3a* forward primer: 5'-cctgaagcctcaagagcagt-3', reverse primer: 5'-tggtctccttctgttctttgc-3', probe number #46 (cat. no. 04688066001); *DNMT3b* forward primer: 5'-ggtgcactgagctcgaaag-3', reverse primer: 5'- aagaggtgtcggatgacagg-3', probe number #3 (cat. no. 04685008001); *OCT4* forward primer: 5'-cttcggatttcgtcttctcg-3', reverse primer: 5'-cttagccaggtccgaggat-3', probe number #22 (cat. no. 04686969001); *AP2*γ forward primer: 5'-gagccaaatcgaaaaatgga-3', reverse primer: 5'-gccaaatgaacagcttcacc-3', probe number #7 (cat. no. 04685059001); *NANOG* forward primer: 5'-atgcctcacacggagactgt-3', reverse primer: 5'-cagggctgtcctgaataagc-3', probe number #69 (cat. no. 04688686001); *TBP* forward primer: 5'-gcccatagtgatctttgcagt-3', reverse primer 5'-cgctggaactcgtctcacta-3', probe number #67 (cat.

no. 04688660001). Rat primers: *Tet1* forward primer: 5'-agagggaaaagaagcccaaa-3', reverse primer: 5'-aacaaacccaacctttgctc-3', probe number #60 (cat. no. 04688589001); *Ezh2* forward primer: 5'-gactggtgaagagttgttctttga-3', reverse primer: 5'-ctcgttcgatgcccacata-3', probe number #122 (cat. no. 04693566001), Dnmt3a forward primer: 5'-aacggaagcgggatgagt-3', reverse primer: 5'-gcaatcaccttggctttctt-3', probe number #75 (cat. no. 04688988001); Dnmt3b forward primer: 5'-caaatccagggacttgcag-3', reverse primer: 5'-acccactagcaccctcttctt-3', probe number #94 (cat. no. 04692110001).

H3K27me3 ELISA

Treated NT2 cells (n=3 experiments, each in triplicate) were collected and the crude histones were extracted using the trimethylated lysine 27 at histone 3 (H3K27me3) ELISA kit (Active motif, Belgium) according to the manufacturer's instructions. ELISA plates were already pre-coated with an H3 antibody. H3K27me3 standards and the extracted crude histones from each sample were added to the appropriate well. After incubation, the primary antibody was added and incubated for 1h. A secondary HRP conjugated antibody was then added followed by incubation for 1h. The colorimetric reaction was then initiated with a developing solution, which reacts with the HRP conjugate and produces a colorimetric signal. The reaction was stopped after 5 mins by adding a stop solution to all wells. All samples were determined in duplicate. Absorbance was measured using a spectrophotometer (LabSystems; 450nm wavelength). Known H3K27me3 standards were used to construct a standard curve, which was used to calculate total H3K27me3 per well. Total H3K27me3 was expressed relative to the total protein concentration, which was determined by the Bradford method (Thermo Fisher).

Statistics

For human fetal gonad culture and xenograft studies, results were analyzed by two-way ANOVA to account for inter-individual variation between fetuses and xenograft replicates from the same fetus, as previously described *(55)*. The same method was used for NT2 cell studies. For rat in vivo and in vitro studies, analysis was performed using unpaired, 2-tailed t-tests. $P < 0.05$ was used to determine significance.

Ethics statement

Human fetal testes were obtained after elective termination of pregnancy, according to the Declaration of Helsinki–Ethical Principles for Medical Research Involving Human Subjects.

Ethical approval for the study was obtained from the South East Scotland Research Ethics Committee (reference number LREC08/S1101/1). Women gave written informed consent. Animal studies received specific approval by the UK Home Office (PPL 60/4564), including ethical approval, and were performed according to the Animal (Scientific Procedures) Act 1986.

Author contributions:

Conceived and designed the experiments: P.H.G., R.M.S., R.A.A. and R.T.M. Performed the experiments: P.H.G., S.v.D., J.McD., A.J., C.McK., S.Mcp. K.K, Analyzed the data: P.H.G., S.v.D., R.T.M., J.McD., R.M.S. Contributed reagents/materials/analysis tools: R.T.M., R.M.S., and R.A.A. Wrote the paper: P.H.G., R.M.S., R.T.M., R.A.A. All authors approved the submitted version.

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Figure Legends

Figure 1

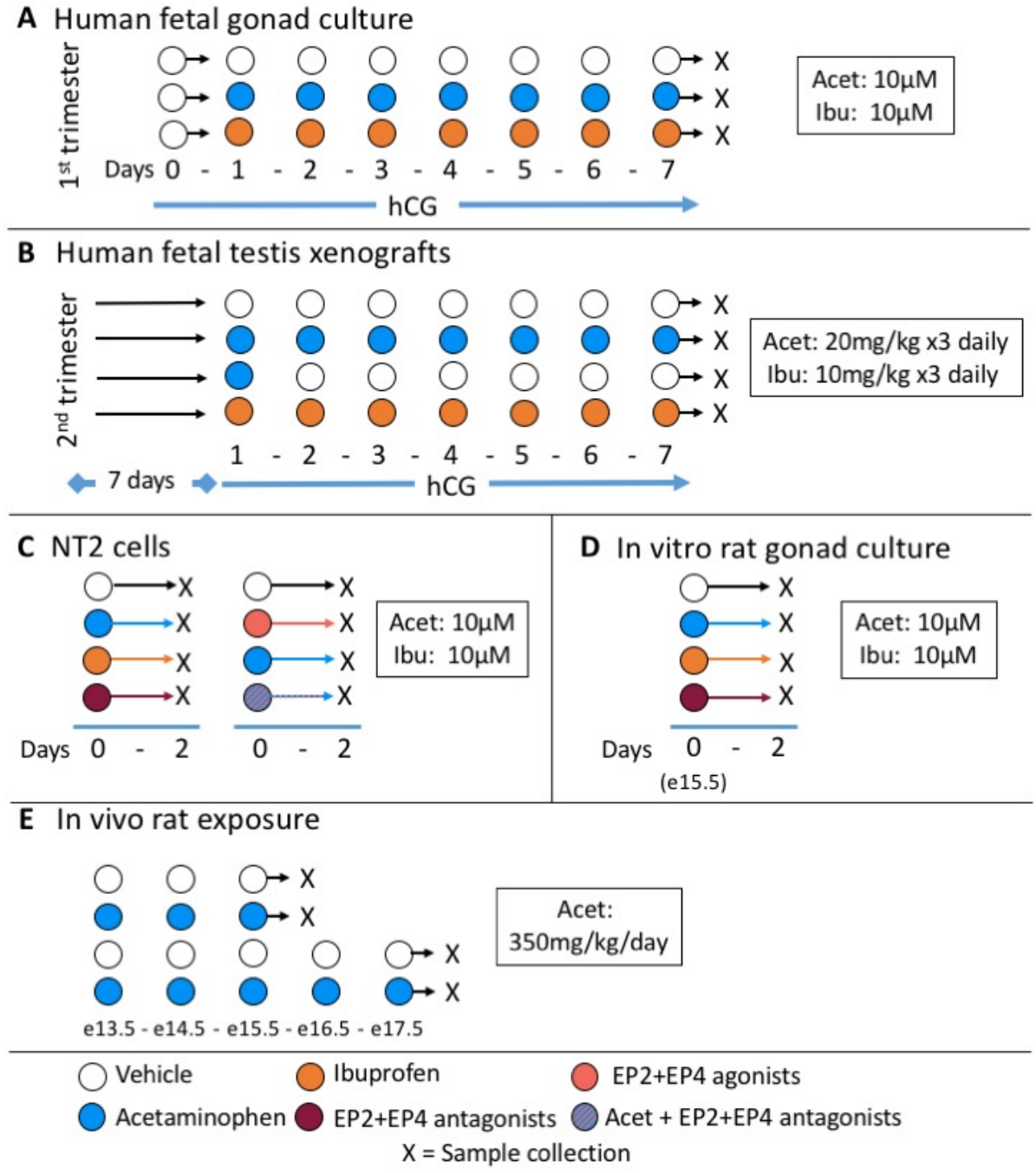


Figure 1. Exposure/treatment regimens and the different investigative models used in the present studies. Each panel shows a different experimental model together with the exposure/treatment (coloured circles) regimen employed, including timescale. **A** hanging drop culture of 1st trimester human fetal testes and ovaries. **B** xenografts of 2nd trimester human fetal testes grafted into nude mice. Note experimental treatments commenced 7 days after grafting in order to allow

vascularisation of the grafts. **C** NTERA-2 (NT2; testicular germ cell tumor cell line) cell culture. **D** hanging drop culture of fetal (e15.5) rat testes and ovaries. **E** in vivo exposure of fetal rat testes and ovaries by treatment of pregnant rats (e15.5 and e17.5). Note that the order of panels corresponds with the order of models presented in Results.

Figure 2

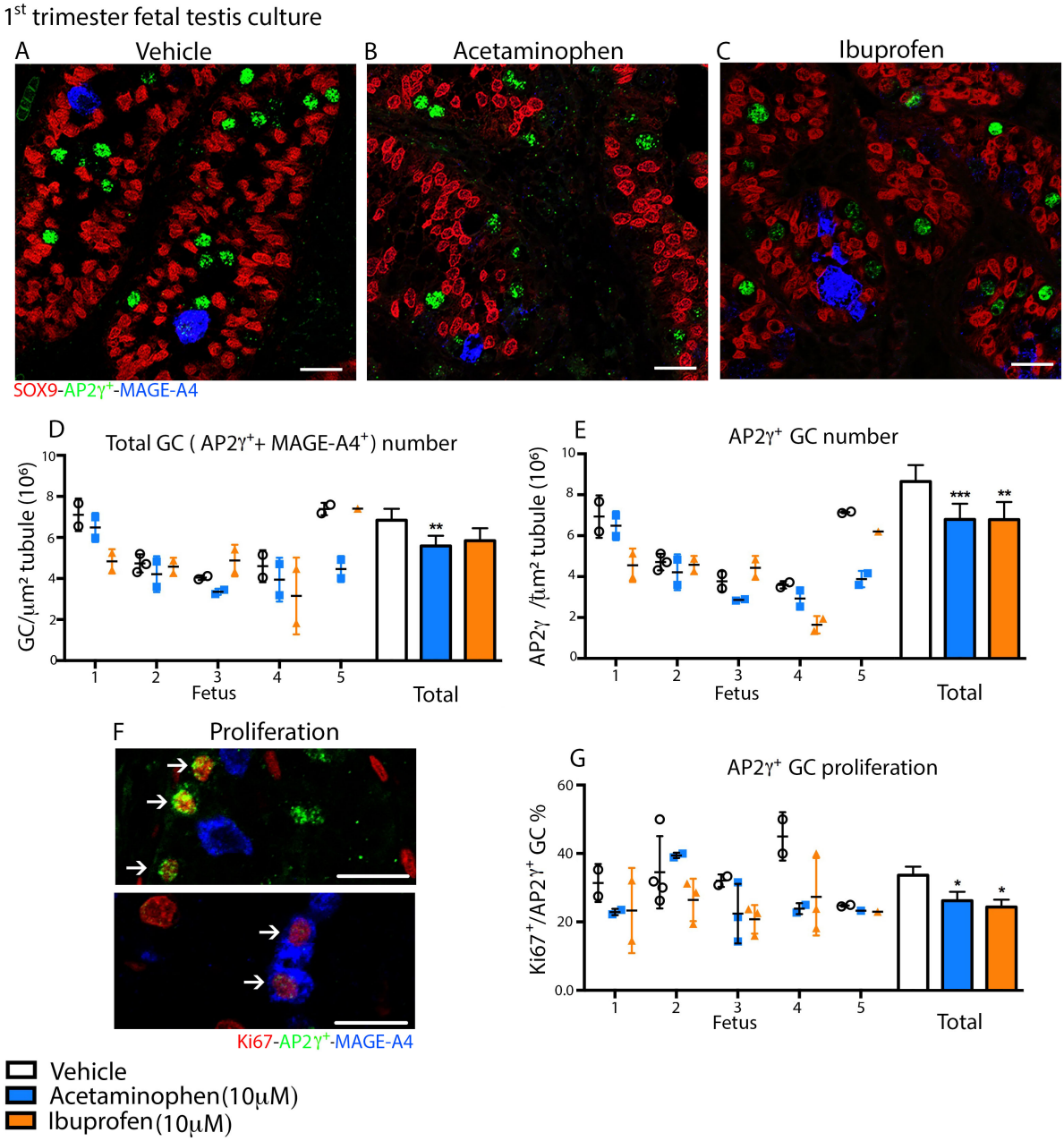


Figure 2. Effect of analgesic exposure of 1st trimester fetal human testis tissue for 7 days in hanging drop culture on germ cell (GC) number. Testis tissue pieces (~1mm^3) from 5 fetuses (8-11 gestational weeks) were cultured for 7 days with vehicle, acetaminophen (10μM) or ibuprofen (10μM). Tissue was immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC -

blue) as depicted in panels **A**-**C**. Scale bar, 20μm. Individual data points represent counts from a single tissue piece in panels **D** and **E** together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total). Panel **F** shows an example of a testis tissue section from a vehicle-exposed sample, triple-immunostained for the proliferation marker Ki67 (red) and the GC markers AP2γ (green) and MAGE-A4 (blue). White arrows refer to proliferative GC showing double staining for Ki67 and AP2γ (upper panel) or MAGE-A4 (lower panel). Quantification of the % of proliferative (Ki67$^+$) AP2γ^+ GC from the different treatment groups is shown in panel **G.** Data analysed by 2-factor ANOVA (*p<0.05, **p<0.01, ***p<0.001 versus corresponding vehicle-exposed group).

Figure 3

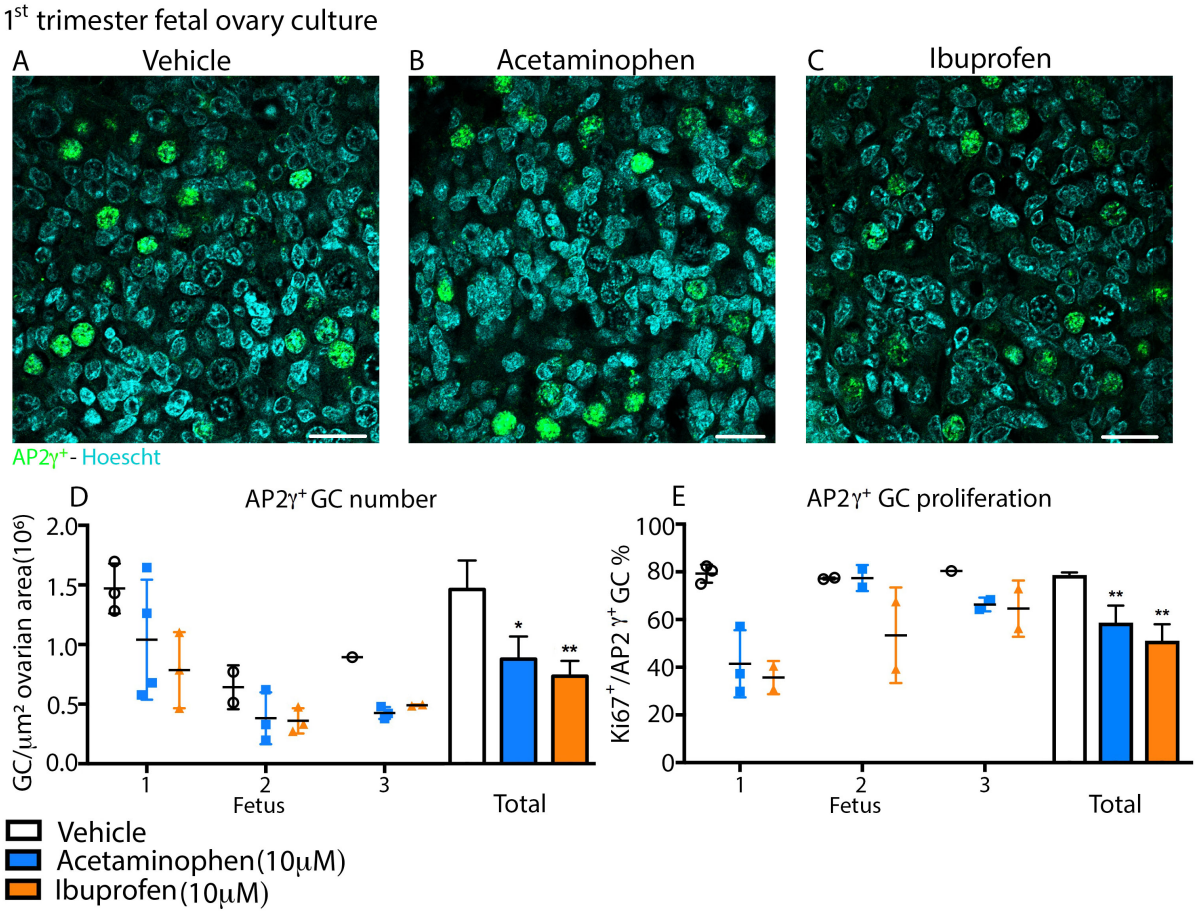


Figure 3. Effect of analgesic exposure of 1st trimester fetal human ovary tissue for 7 days in hanging drop culture on germ cell (GC) number. Ovary tissue pieces (~1mm^3) from 3 fetuses (9-11 gestational weeks) were cultured for 7 days with either vehicle, acetaminophen (10μM) or ibuprofen (10μM). Tissue was fixed and double-immunostained for AP2γ (GC - green) and Hoechst (counterstaining - blue) as

depicted in panels **A**-**C**. Scale bar, 20μm. Individual data points represent GC counts (AP2γ^+) from a single tissue piece in panel **D** together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total). Quantification of the proliferative (Ki67$^+$) AP2γ^+ GC from the different treatment groups is shown in panel **E**. Data in **D** and **E** was analysed by 2-factor ANOVA (*$p<0.05$, **$p<0.01$, versus corresponding vehicle-exposed group).

Figure 4

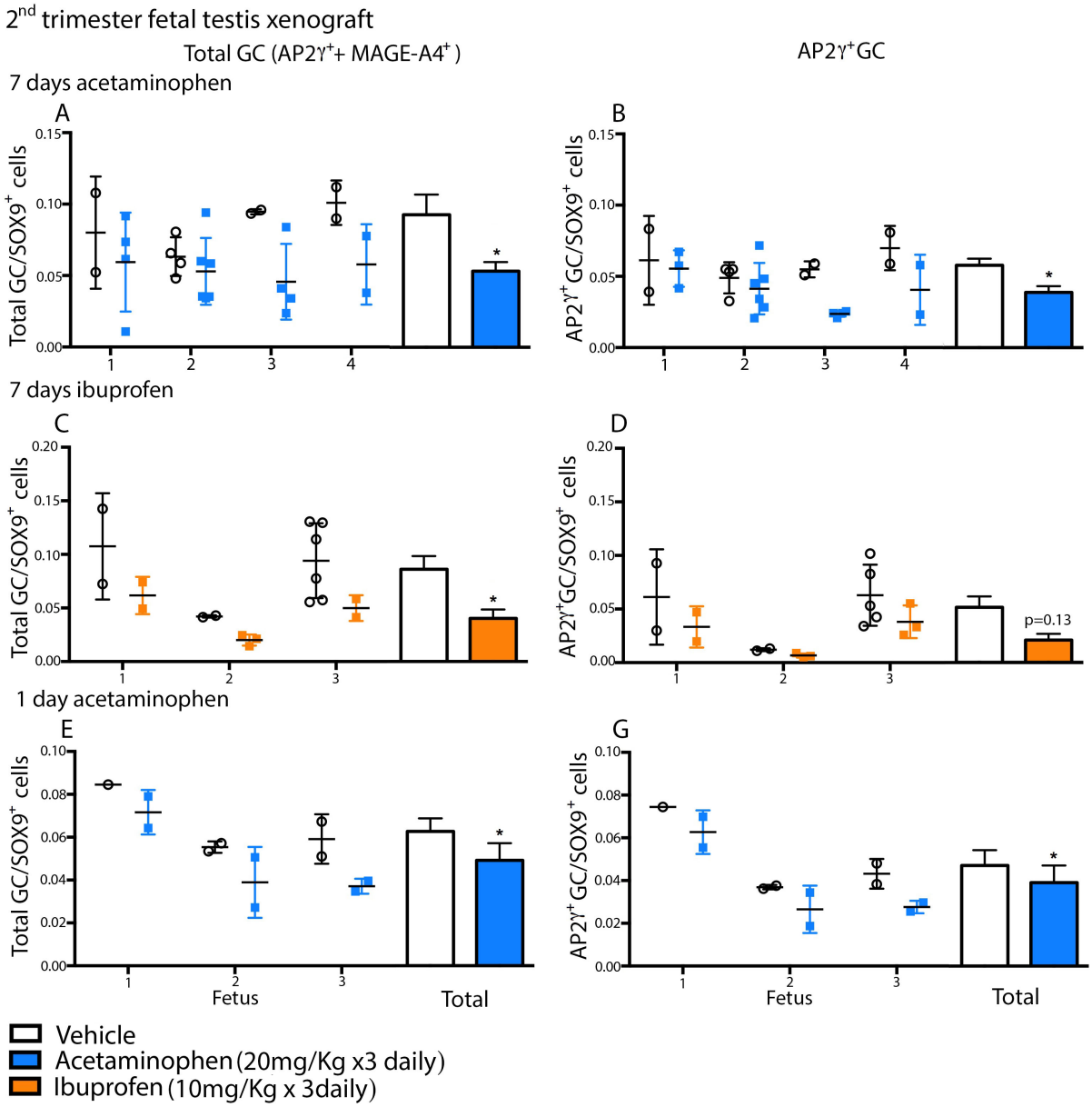


Figure 4. Effect of acetaminophen or ibuprofen exposure of 2nd trimester fetal human testis tissue xenografts on germ cell (GC) number. Testis tissue pieces (~1mm^3) from 4 fetuses (7-day acetaminophen) or 3 fetuses (1-day acetaminophen, ibuprofen), all within 14-17 gestational weeks, were xenografted into nude mice. Host mice were administered vehicle, acetaminophen (20mg/kg x3 per day) or ibuprofen (10mg/kg x3 per day) before xenograft recovery. Tissue was fixed and triple-immunostained for SOX9 (Sertoli cells - red), AP2γ (GC - green) and MAGE-A4 (differentiated GC - blue). Individual data points represent GC counts from a single tissue piece in panel panels **A** and **B** for 7-days acetaminophen exposure, panels **C** and **D** for 1-day acetaminophen exposure and panels **E** and **F** for ibuprofen exposure,

together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA); *p<0.05, versus corresponding vehicle-exposed group. Corresponding MAGE-A4$^+$ GC counts and their % proliferation are shown in figs. S1 and S2 respectively.

Figure 5

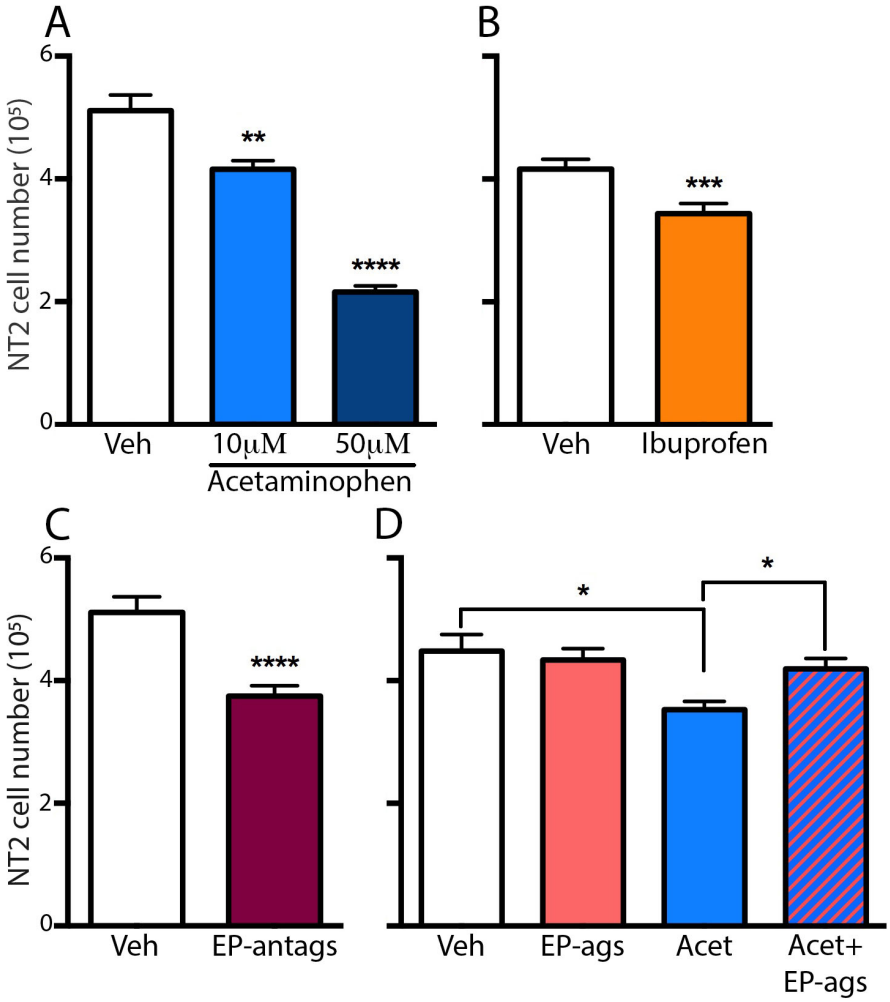


Figure 5. Effect of exposure of NT2 cells to analgesics or prostaglandin E2-receptor modulators on cell number. NT2 cells (2x10^5; n=3-4) were cultured for 48h in medium supplemented with either vehicle (Veh), acetaminophen (10-50μM), ibuprofen (10μM), EP2+EP4 antagonists [EP-antags: 10μM L-161,982 (EP2 antagonist) + 10μM PF04418948 (EP4 antagonist)], or with EP2+EP4 agonists [EP-ags: 10μM Butaprost (EP2 agonist) + 10nM CAY10598 (EP4 agonist)] or the combination of acetaminophen + EP2/EP4 agonists (Acet+EP-ags). The mean ± SEM NT2 cell number for each treatment is shown in panels **A** – **D.** Panel **D** shows that co-culture of NT2 cells with PGE_2 agonists blocks the negative effect of acetaminophen exposure on cell number. Graphs show means ± SEM from three

independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus corresponding vehicle-exposed group. Panel **D** analysis was followed by a comparison between Acet and Acet+EP-ags columns. Corresponding data for NT2 cell death as a consequence of the different treatments are shown in Supplementary figure 3.

Figure 6

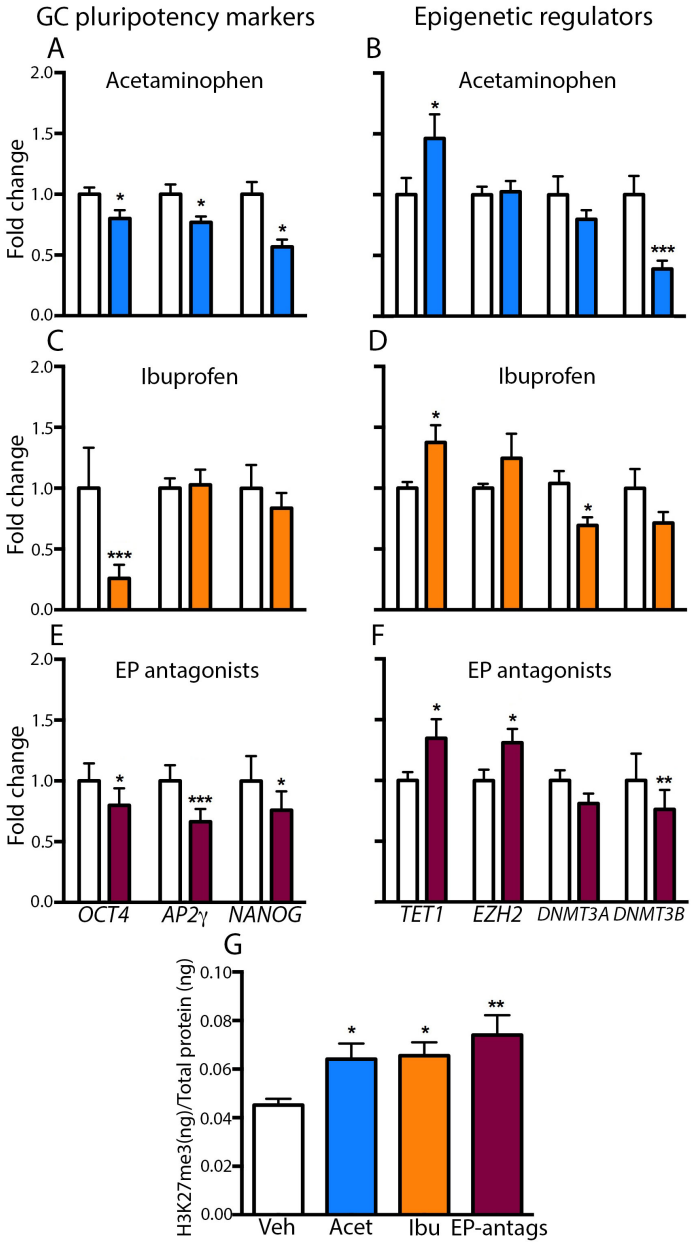


Figure 6. Effect of exposure of NT2 cells to analgesics or prostaglandin E_2 antagonists on mRNA expression of GC pluripotency markers, epigenetic regulatory genes and overall H3K27me3 levels. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with vehicle (Veh), acetaminophen (Acet; 10-50μM), ibuprofen (Ibu; 10μM) or EP2+EP4 antagonists [EP-antags (10 μ M L-161,982; EP2 antagonist + 10 μ M PF04418948; EP4 antagonist)]. Results show mRNA expression relative to the vehicle complementary DNA (open bars) for the GC pluripotency markers *OCT4*, *AP2* γ and *NANOG* (**A, C and E**) and for the epigenetic regulatory genes *TET1*, *EZH2*, *DNMT3a* and *DNMTb* (**B, D and F**) after exposure of NT2 cells to acetaminophen (blue), ibuprofen (orange) or EP2+EP4 antagonists (maroon)

(n=3). Panel **G** shows relative amount of H3K273 present in the NT2 cells after culture with the different treatments. Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, **p<0.01, ***p<0.001, in comparison with corresponding vehicle or vehicle complementary DNA.

Figure 7

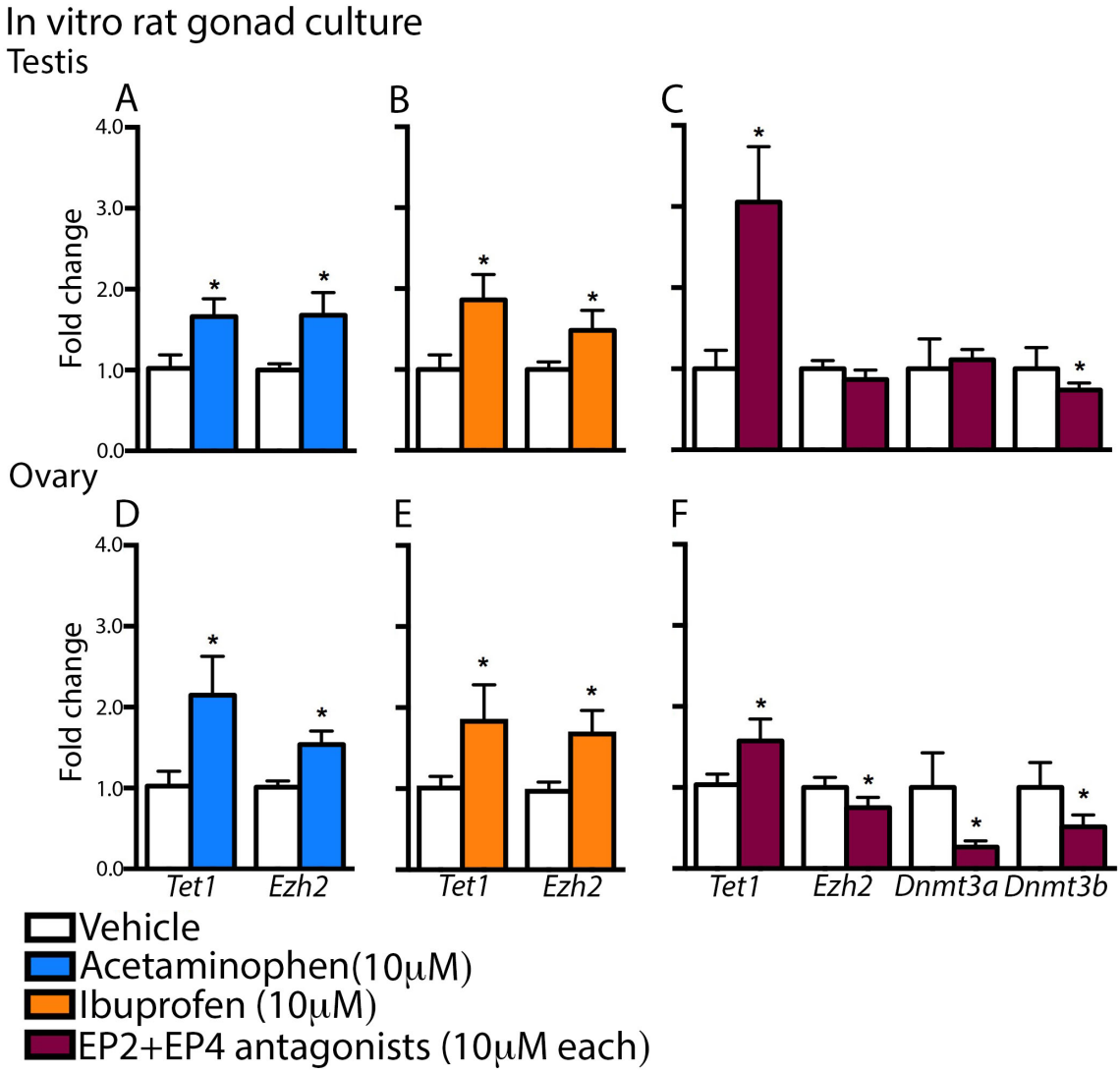


Figure 7. Effect of exposure of rat fetal gonads to analgesics or prostaglandin E_2 antagonists in vitro on mRNA expression of epigenetic regulatory genes. Testis and ovary tissue pieces (~1mm^3) from e15.5 fetal rats were cultured for 48h with either vehicle (open bars), acetaminophen (blue; 10μM), ibuprofen (orange; 10μM) or EP2+EP4 antagonists [Maroon; 10 μ M L-161,982 (EP2 antagonist) + 10 μ M PF04418948 EP4 antagonist)]. Panels **A** to **F** show mRNA expression relative to the vehicle complementary DNA (open bars) for *Tet1*, *Ezh2*, *Dnmt3a* and *Dnmt3b* for

cultured fetal testes (**A** to **C**) or ovaries (**D** to **F**) (Mean ±SEM, n=13-30). Data were analyzed by paired t-test; *p<0.05, in comparison with corresponding vehicle control group.

Figure 8

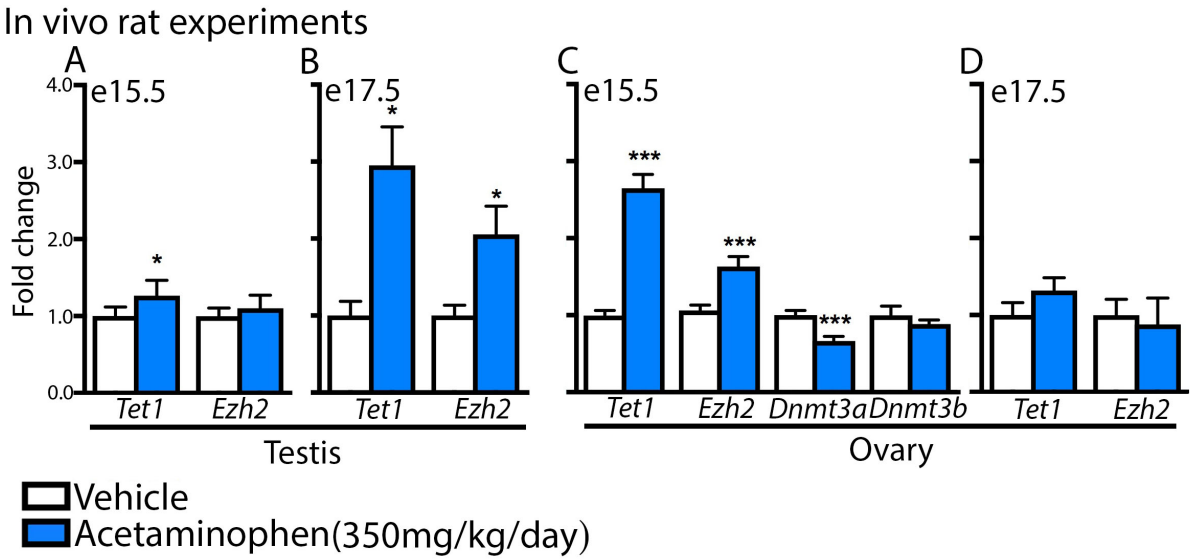
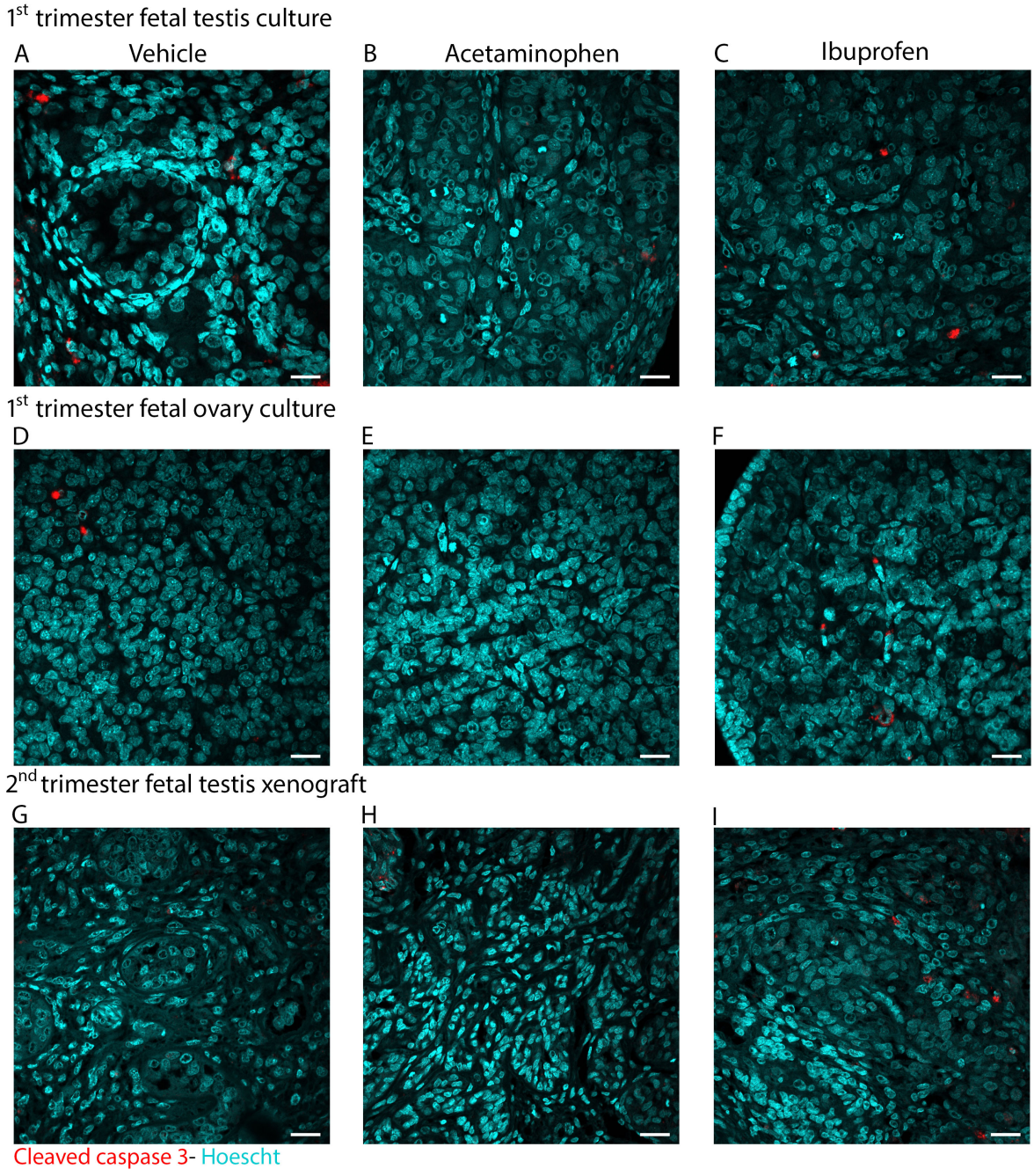


Figure 8. Effect of exposure of pregnant rats to acetaminophen on mRNA expression of epigenetic regulatory genes in fetal testes and ovaries. Pregnant rats were administered acetaminophen (350mg/kg/day x1), commencing on e13.5, and fetal gonadal tissue was collected on e15.5 or e17.5, 3 hours after the final maternal treatment. Panels **A** to **D** show mRNA expression relative to the vehicle cDNA for the same genes after acetaminophen exposure in vivo for e15.5 (**A**) and e17.5 (**B**) testes and for e15.5 (**C**) and e.17.5 (**D**) ovaries (Means ± SEM n=7-25). Data were analyzed by two-tailed, unpaired t-test; *p<0.05, ***p<0.001, in comparison with corresponding vehicle control group.

Supplementary figure legends

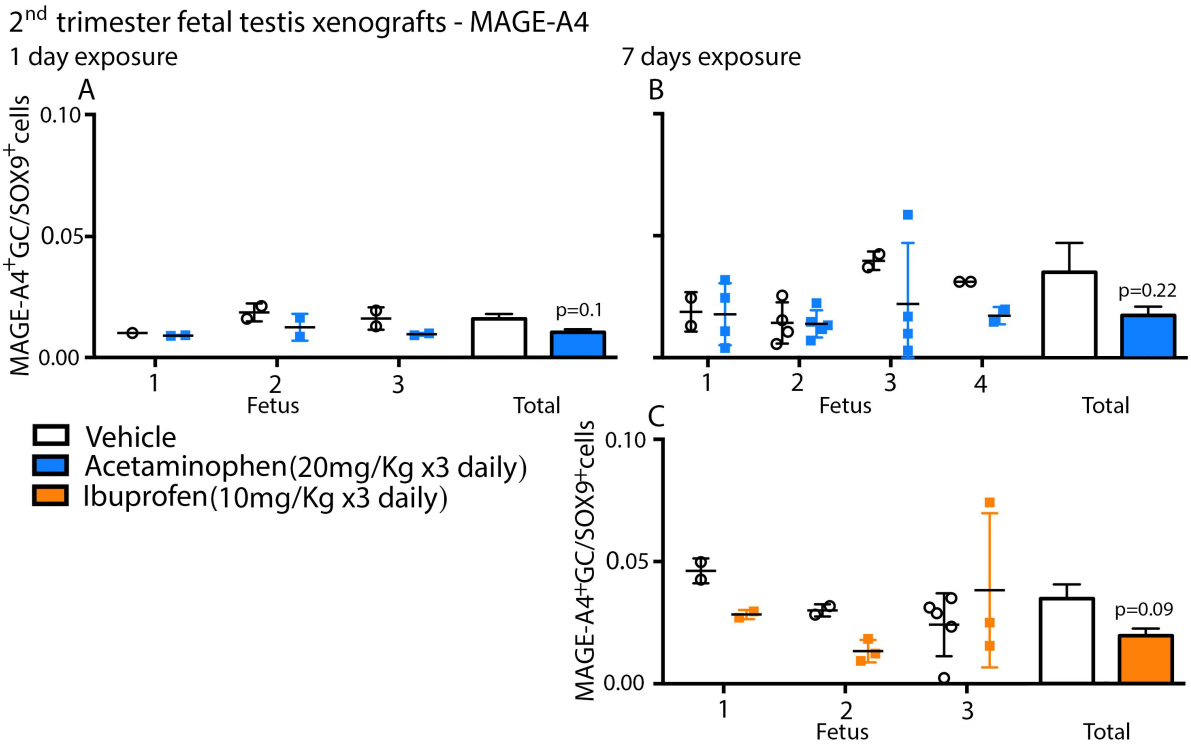
Supplemental figure 1



Supplemental figure 1. Effect of analgesic exposure on apoptosis. Apoptosis as a consequence of analgesic exposure was studied in 3 different systems: 1st trimester fetal testis (A-C) and ovary (D-F) culture, and 2nd trimester fetal testis xenograft (G-I) exposed to human relevant doses of acetaminophen and ibuprofen.

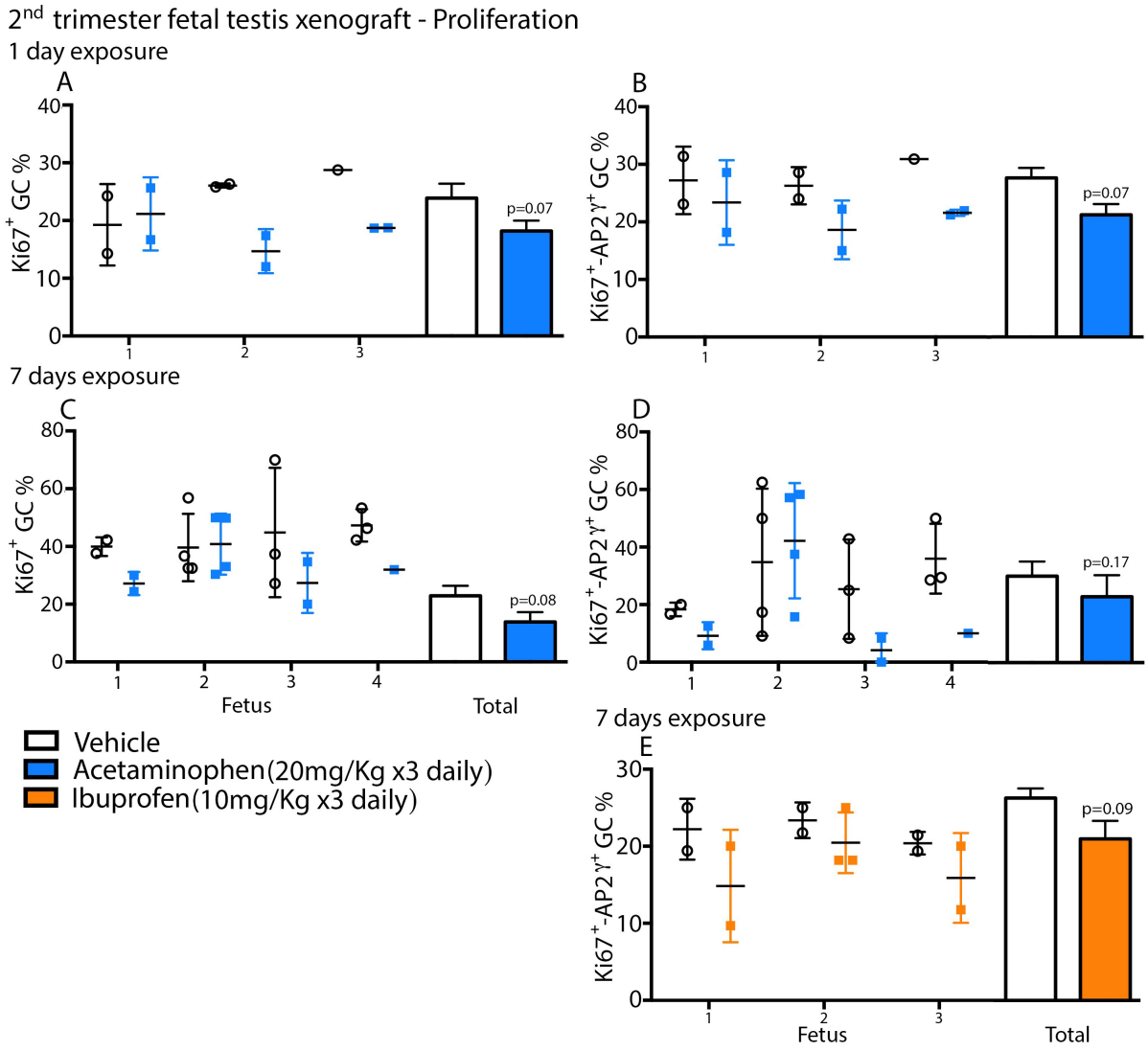
Tissue was immunostained for Cleaved caspase 3 (red) and Hoescht (counterstaining - blue). Scale bar, 20μm.

Supplementary figure 2



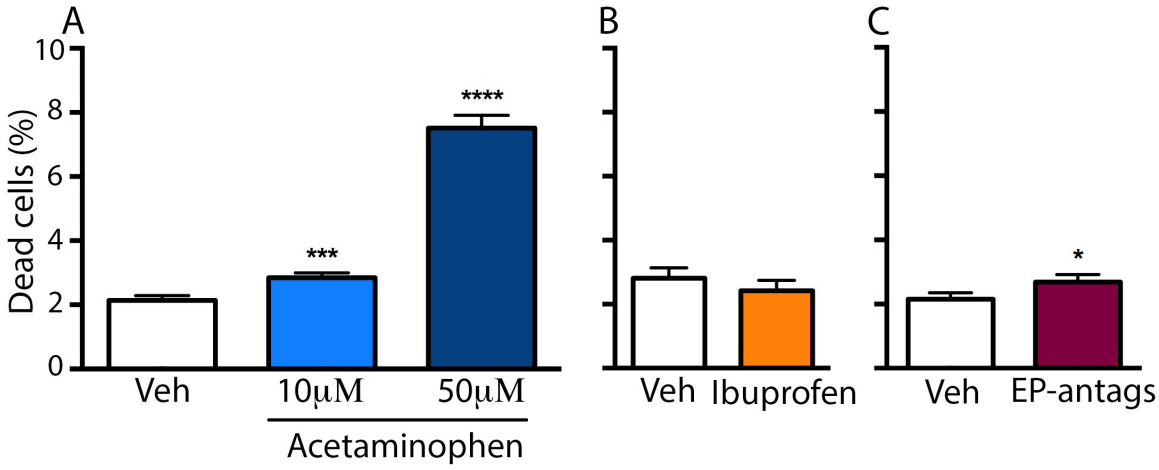
Supplemental figure 2. Effect of acetaminophen or ibuprofen exposure of 2nd trimester fetal human testis tissue xenografts on MAGE-A4$^+$ germ cell (GC) number. Testis tissue pieces (~1mm^3) from 4 fetuses (7-day acetaminophen), 3 fetuses (1-day acetaminophen) or 3 fetuses (ibuprofen) were xenografted into nude mice. Host mice were administered vehicle, acetaminophen (20mg/kg x3 per day) or ibuprofen (10mg/kg x3 per day) before xenograft recovery. Tissue was fixed and triple-immunostained for Ki67 (proliferating cells) and MAGE-A4 (subpopulations of GCs). MAGE-A4$^+$ GC counts for each tissue piece for each fetus for each treatment are shown by the individual symbols in panels **A** and **B** and **C** for 1 day acetaminophen, 7 days acetaminophen and 7 days ibuprofen respectively, together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA).

Supplemental figure 3



Supplemental figure 3. Effect of acetaminophen or ibuprofen exposure of 2nd trimester fetal human testis tissue xenografts on germ cell (GC) proliferation. Further results obtained from the exposure of 2nd trimester human testis xenografts to acetaminophen or ibuprofen. Quantification of the % of proliferative GC ($Ki67^+$) after 1 day and 7 days exposure to acetaminophen are respectively shown in panels **A** and **C**. Quantification of the % of proliferative ($Ki67^+$) $AP2\gamma^+$ GC after 1 day acetaminophen, 7 days acetaminophen or 7 days ibuprofen are respectively shown in panels **B, D** and **E** together with the mean ± SEM for each fetus/treatment; the overall mean ± SEM for each treatment group is also shown (Total) together with the results of statistical analysis (2-factor ANOVA).

Supplemental figure 4



Supplemental figure 4. Effect of exposure of NT2 cells to analgesics or prostaglandin E2-receptor modulators on cell death. NT2 cells (10^5; n=3) were cultured for 48h in medium supplemented with either vehicle (Veh), acetaminophen (10-50μM), ibuprofen (10μM) or EP2+EP4 antagonists [EP-antags: 10 μ M L-161,982 (EP2 antagonist) + 10 μ M PF04418948 (EP4 antagonist)]. The % of NT2 dead cells at the end of the experiment for each treatment is shown in panels **A** – **C.** Graphs show means ± SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA; *p<0.05, ***p<0.001, ****p<0.0001 versus corresponding vehicle-exposed group.